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Rebecca Greenwald

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Abstract

Title of dissertation:

The Role of B7 Ligand Interactions During an *in vivo* Type 2 Immune Response

Rebecca Greenwald

Doctor of Philosophy, 1998.

Dissertation directed by:

William C. Gause, Ph.D.

Professor, Department of Microbiology and Immunology

The nematode parasite, *H. polygyrus*, has been extensively used as a model to study the type 2 *in vivo* immune response. This response is characterized by the development of IL-4 producing T cells which mediate the development of other allergy-associated effector cell populations. Following oral inoculation with *H. polygyrus* larvae, the host rapidly develops a type 2 immune response characterized by intestinal mastocytosis, blood eosinophilia, elevations in T cell IL-2R expression, *in situ* $CD4^+$ T cell expansion and IL-4 production, and increases in B cell MHC class II expression, MLN GC formation and serum IgE and IgG1 levels.

The costimulatory signal provided to naïve T helper (Th) cells through CD28/CTLA-4 interactions is required for *in vivo* Th cell effector function associated with cytokine production. In this project, I examined whether the two well-characterized

ligands for these molecules, B7-1 and B7-2, differentially influence the development of a type 1 or type 2 immune response. Administration of the combination of anti-B7-1 and anti-B7-2 mAbs inhibited *H. polygyrus*-induced increases in serum IgG1 and IgE levels, the expansion of mesenteric lymph node (MLN) germinal centers, in situ $CD4^+$ T cell expansion, elevated blood eosinophils, and increased intestinal mucosal mast cells. Similarly, both Abs blocked MLN and Peyer's Patch's patch cytokine gene expression and elevations in MLN T cell derived IL-4 protein secretion. However, in the same experiments, administration of either anti-B7-1 and anti-B7-2 mAbs alone had little effect on these parameters. T cell and B cell activation was also blocked by the combination of anti-B7-2 and a B7-1 specific mutant Y100F CTLA-4Ig construct. These results suggest that, to the extent that anti-B7-1 and anti-B7-2 mAbs block B7 interactions, either B7-1 or B7-2 ligand interactions can provide the required costimulatory signals that lead to T cell effector function during the type 2 *in vivo* immune response.

Given that other studies suggested that B7-2 can favor a type 2 immune response, we examined the *H. polygyrus* immune response in B7-2KO mice. Our results in *H. polygyrus*-inoculated B7-2KO mice confirm that B7-2 is not required at the initiation of the immune response. As the type 2 immune response develops, however, B7-2 has an increasingly important role in effector cell functions. In *H. polygyrus*-inoculated B7-2KO mice, elevations in T cell derived IL-4 production and IgE secretion by B cells are blocked, while secretion of IgG1 by B cells and GC formation remain intact. These results demonstrate differential B7-2 dependence of effector cells associated with the type 2 immune response.

The role of CD28 and CTLA-4 were also examined during the development of a type 2 immune response. The T cell surface molecule, CD28, is widely considered to be the principle costimulatory molecule involved in T cell differentiation to effector function, although this has been difficult to directly examine *in vivo*. Our results show that in CD28KO mice, elevations in IL-4 gene expression and protein secretion are blocked during the systemic immune response to goat anti-mouse IgD, and associated increases in serum IgG1 and IgE are also inhibited compared to untreated control levels. In marked contrast, T cell differentiation to IL-4 production is comparable in CD28KO and CD28WT *H. polygyrus*-inoculated mice, and elevations in both serum IgG1 and IgE levels occur. These results indicate that the specific kind of type 2 immune response determines whether T cell differentiation to IL-4 production can occur in CD28 deficient mice.

Since *H. polygyrus*-inoculated CD28KO mice develop a strong type 2 immune response, CTLA-4Ig was administered at the initiation of this response to determine whether an alternative B7 ligand, probably CTLA-4, could provide the required costimulatory signal. Although our results consistently demonstrated a vigorous type 2 immune response in *H. polygyrus*-inoculated CD28KO mice, conflicting results were obtained after CTLA-4Ig or anti-CTLA-4 Ab administration to inoculated mice. Given the recent availability of blocking CD28 and CTLA-4 Abs, our studies resolved this paradox by administering the combination of anti-CD28 and/or anti-CTLA-4 Abs to *H. polygyrus*-inoculated, wild-type mice. These results demonstrate that in CD28WT mice, CD28 is necessary for T cell differentiation to effector function in the *H. polygyrus* response and that CTLA-4 is also required for an optimal response.

The last section of this project considers the role of B7 ligand interactions in the induction of T cell memory. Our results demonstrate that CTLA-4Ig administration during the primary immune response to *H. polygyrus* does not influence the subsequent host protective challenge secondary response as measured by adult worm counts, egg production, GC formation, and serum IgE levels. These studies indicate that blocking B7 ligand interactions may only transiently block the immune response and therefore, is a critical consideration for the design of therapeutic interventions.

Dedication

To my family for sharing a lively enjoyment of life.

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The Role of B7 Ligand Interactions During an *in vivo* Type 2 Immune Response

Rebecca Greenwald

Dissertation submitted to the Faculty of the Department of Microbiology and Immunology Graduate Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the Degree of Doctor of Philosophy, 1998.

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List of Abbreviations

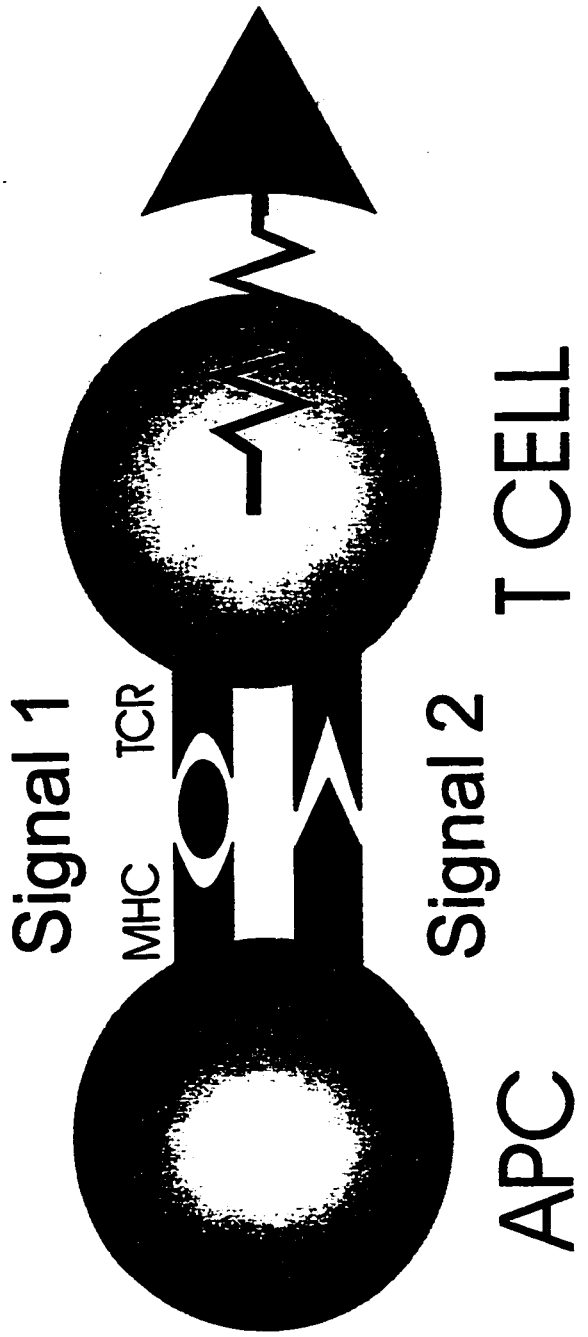
aa, amino acid; Ab, antibody; Ag, antigen; APC, antigen presenting cell; BCIP, 5-bromo-4-chloro-3-indolyl-phosphate; bp, base pair; CD40L, CD40 ligand; Con A, concanavalin A; CTL cytotoxic T lymphocyte; CTRL, control; CY5, cyanine-chrome 5; dNTP, deoxynucleoside triphosphate; EAE, experimental allergic encephalomyelitis; FACS, fluorescent activated cell sorter; FBS, fetal bovine serum; FSC, forward light scatter; FITC, fluorescein-5-isothiocyanate; GaMIgD; goat anti-mouse IgD; HBSS, Hank's balanced salt solution; HP, *Heligosomoides polygyrus*; HPRT, hypoxanthine-guanine ribosyl transferase, IFN-γ, interferon gamma; IgC, immunoglobulin constant; IgV, immunoglobulin variable; IL-2R, IL-2 receptor; LPS, lipopolysaccharide; mAb; monoclonal antibody; MLN, mesenteric lymph node; NK, natural killer; NOD, non-obese diabetic; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PE, phycoerythrin; PNA, peanut agglutinin; PP, Peyer's patch; RT, reverse transcriptase; RT-PCR, reverse transcription-polymerase chain reaction; SA-PE, stepavidin-phycoerythrin; SE, standard error; sRBCs, sheep red blood cells; TCR, T cell receptor, Th, T helper; Th1, T helper 1; Th2, T helper 2.

I. Introduction

A. General introduction to *in vivo* immune responses

Understanding the specific cellular interactions required for activation of the host immune response to an infectious pathogen is currently a major focus of study in Immunology. Particularly important at the initiation of the host adaptive immune response is the activation of naïve T helper cells (Th) through interactions with antigen presenting cells (APCs). As lymph travels from the site of infection to the secondary lymphoid tissues, foreign antigen (Ag) is endocytosed by APCs, which include B cells, dendritic cells and macrophages. APCs present the foreign antigen in the form of antigenic peptide bound to major histocompatibility complex (MHC) class II molecules. In the T cell rich regions of the secondary lymphoid tissues, APCs, usually dendritic cells (Sousa et al., 1997), interact with naïve $CD4^+$ T cells and initiate T cell activation. T cell activation leading to cytokine production and helper effector function requires two distinct signaling interactions from APCs (Figure 1) (Bretscher and Cohn, 1970). The first signal is delivered through the engagement of cell surface MHC class II molecules bound to antigenic peptide with an antigen-specific T cell receptor (TCR). A second non-antigen-specific signal is transmitted through costimulatory molecules. Numerous studies have shown that the costimulatory receptor:ligand interactions important for T helper cell activation leading to effector function include B7-1 and B7-2 (expressed on APCs) interactions with either CD28 or CTLA-4 (expressed on Th cells) (Figure 2)

Figure 1. Two signals are required for the activation of naïve $CD4^+$ T cells leading to cytokine production and effector Th cell function. The first signal is delivered through the antigen specific TCR which interacts with MHC class II molecules presenting antigenic peptides on the surface of APCs. A second, non-antigen specific signal is delivered through costimulatory molecules.


Signal 1
MHC
TCR
Signal 2
T CELL
APC

(June et al., 1990; June et al., 1994). T cell surface CD28 provides a potent costimulatory signal following interactions with the B7 molecules, B7-1 and B7-2. Its homologue, CTLA-4, also binds B7 molecules, but signaling through CTLA-4 can, at least under some circumstances, provide a negative signal down-regulating T cell activation as observed in CTLA-4KO mice, which exhibit severe lymphoproliferation (Tivol et al., 1995; Waterhouse et al., 1995). Costimulatory molecules may influence T cell differentiation quantitatively, by determining the extent of T cell activation, and qualitatively, by influencing the cytokines that are produced by T helper cells once they become activated. Understanding the effects of costimulatory molecules on T helper cell activation during the course of an *in vivo* immune response could aid in the development of immunotherapies directed at the induction of T cell unresponsiveness in the treatment of allergy, autoimmune disease, and transplantation. This dissertation will focus on the role of the B7-CD28/CTLA-4 costimulatory molecules in the development of a host immune response to the nematode parasite, *H. polygyrus*.

B. Type 1 versus Type 2 immune responses

Effector $CD4^+$ Th cells have been generally subdivided into two groups depending on their cytokine profile: Th1 (type 1 helper) cells secrete interferon-γ (IFN-γ), IL-2, and lymphotoxin, whereas Th2 (type 2 helper) cells secrete IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 (Figure 3) (Mosmann et al., 1986; Mosmann and Coffman, 1989). The corresponding type 1 and type 2 responses involve non-T cell populations that also

Figure 2. B7-1 and B7-2 on APCs (dendritic cells, B cells, and macrophages) interacts with CD28 and CTLA-4 on Th cells. In addition to antigen specific TCR ligation, naïve $CD4^+$ T cells require costimulatory interactions for activation leading to effector function. The interactions between B7-1 and B7-2, and CD28 and CTLA-4 are thought to provide these required costimulatory signals.

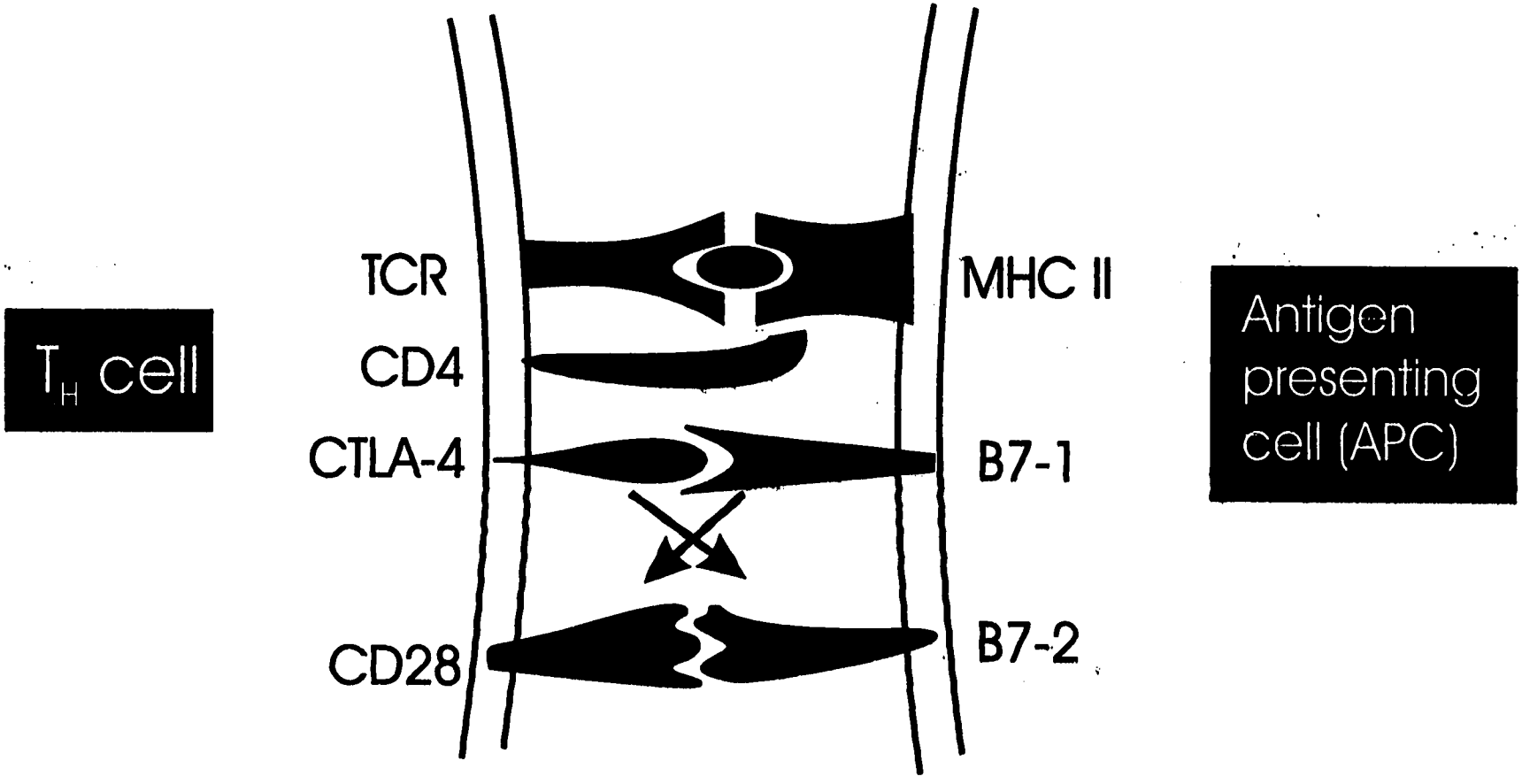
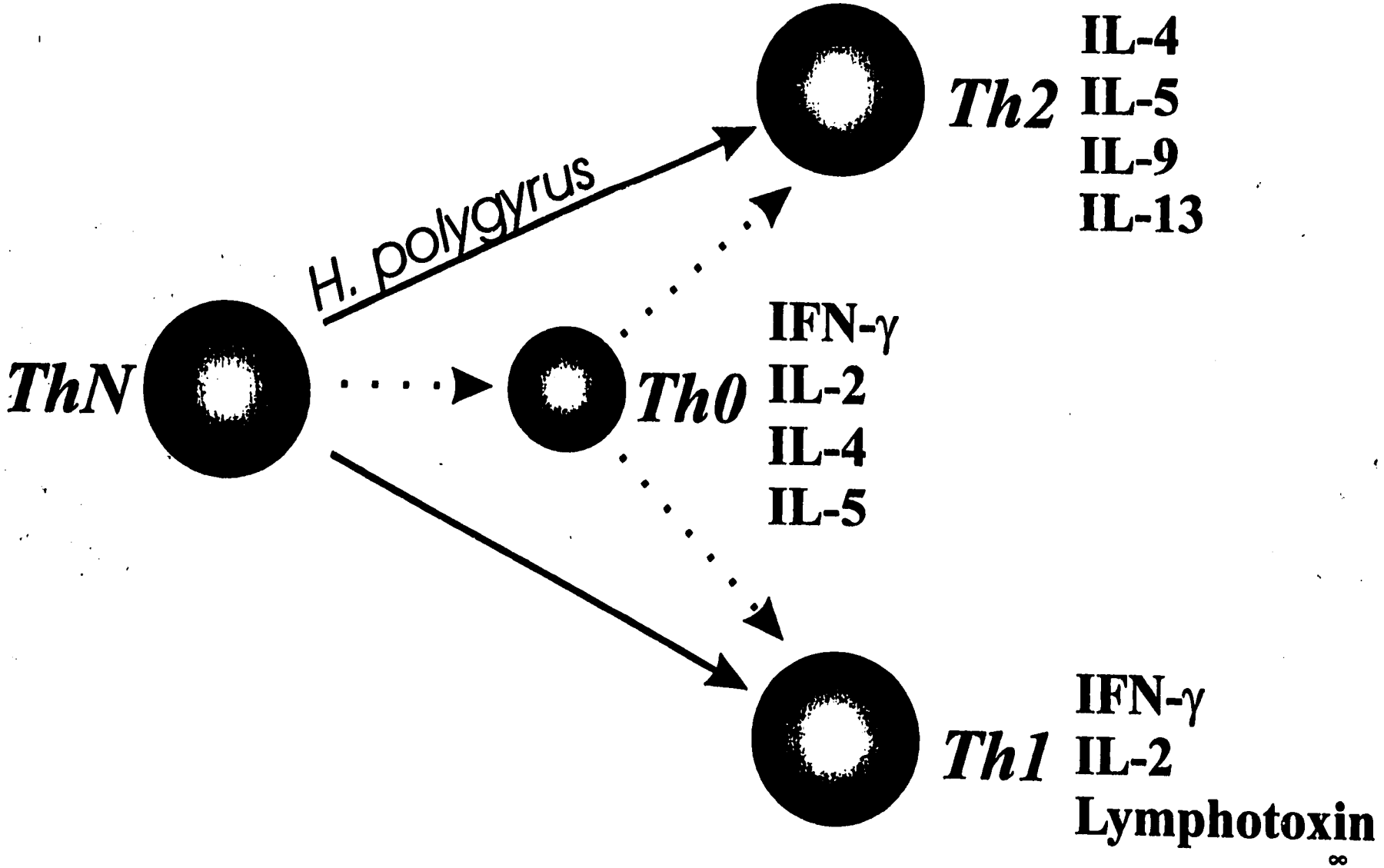

T_H cell
TCR
CD4
CTLA-4
CD28
MHC II
B7-1
B7-2
Antigen presenting cell (APC)

Figure 3. The capacity of naïve Th cells to differentiate into type 1 or type 2 effector cells depends on the particular antigen involved. Naïve $CD4^+$ Th cells rapidly proceed towards a Th2 phenotype following *H. polygyrus* inoculation and do not proceed through a Th0 phenotype. Th1 cells are characterized by elevations in IFN-γ, lymphotoxin, and IL-2 production, while Th2 cells secrete predominately IL-4, IL-5, IL-6, IL-9 and IL-13.


H. polygyrus
ThN
Th0
IFN-γ
IL-2
IL-4
IL-5
Th2
IL-4
IL-5
IL-9
IL-13
Th1
IFN-γ
IL-2
Lymphotoxin

produce these cytokines. In addition to a distinct cytokine profile, the type 1 immune response is associated with an inflammatory response which includes: 1) natural killer cell (NK) activity; 2) macrophage activation; and 3) serum IgG2a elevations. This IFN-γ dominant response is primarily host protective against bacteria, viruses, and intracellular parasites. In contrast, the type 2 immune response is marked by: 1) eosinophilia; 2) mast cell activation; and 3) serum IgE and IgG1 elevations. This IL-4 dominant immune response is host protective against helminthic parasites and is also associated with immediate hypersensitivity reactions (Finkelman et al., 1991; Finkelman et al., 1990).

Depending on the particular antigen involved, the development of type 1 or type 2 effector cells can mediate either a host protective or harmful response. During the immune response to the intracellular parasite, *Leishmania major*, type 2 effector cells do not promote resolution of infection, while type 1 effector cells resolve infection (Heinzel et al., 1989; Heinzel et al., 1991). In contrast, type 2 effector cells mediate a host protective response against helminthic parasites, but these effector cells are also involved in harmful allergic responses. If costimulatory molecules can influence whether a type 1 or a type 2 cytokine response develops, then targeting of these cell surface molecules may be useful for manipulation of T cell cytokine production and effector function. This is an important capability for the development of immunotherapies, which could redirect a harmful immune response towards a host protective response.

C. B7-1 and B7-2

B7-1 (CD80) and B7-2 (CD86) are expressed on the surface of B cells, dendritic cells, macrophages, and T cells (Inaba et al., 1994; Hathcock et al., 1994; Rattis et al., 1996). Both B7-1 and B7-2 are members of the immunoglobulin (Ig) gene superfamily and consist of an Ig variable (V)-like and an Ig-constant 2 (C2)-like extracellular domain (Freeman et al., 1993a; June et al., 1993). The overall homology between murine B7-1 and B7-2 is approximately 25%, with greater homology in the extracellular domain than in the cytoplasmic domain (Freeman et al., 1993c). Although it is possible that crosslinking of cell surface B7-1 or B7-2 may influence APC activation, no direct evidence supports this yet, and the lack of homology between B7-1 or B7-2 cytoplasmic domains among species suggests that the cytoplasmic domain may not provide important cell signaling functions.

The kinetics, pattern of expression, and binding affinities of B7-1 and B7-2 are distinct during an immune response. Stimulation of APCs via CD40:CD40L interactions promotes the expression of B7-1 and B7-2 (Lu et al., 1996). Although several studies suggest that upregulation of B7-2 precedes B7-1 expression on B cells and dendritic cells cultured *in vitro* (Hathcock et al., 1994), few studies have examined the expression of these cell surface molecules on professional APCs during the course of different immune responses *in vivo*. Both B7-1 and B7-2 bind to CTLA-4 with at least a 10-fold higher affinity than to CD28 (Van der Merwe et al., 1997). The dissociation kinetics of the two B7 molecules is different. B7-1 has a slower "off" rate than B7-2 (Linsley et al., 1994). The different kinetics, expression, and binding affinities of B7-1 and B7-2 may result in

distinct functional roles for each of these molecules and account for how differential signaling through B7-1 or B7-2 can vary in different types of *in vivo* immune responses.

D. *In vivo* models of differential signaling through B7-1 versus B7-2

Several groups have examined the differential effects of blocking B7-1 or B7-2 in various model systems. Some reports have suggested that B7-1 and B7-2 can mediate equivalent costimulatory signals *in vitro* (Levine et al., 1995; Lanier et al., 1995; Ueda et al., 1995), while other *in vitro* studies have suggested that T cell derived IL-4 production is particularly dependent on B7-2 signaling (Freeman et al., 1995; Ranger et al., 1996). Administration of blocking B7-1 or B7-2 mAbs during *in vivo* immune responses has yielded different results depending on the system involved (Kuchroo et al., 1995; Racke et al., 1995; Lenschow et al., 1995). In one murine model of autoimmunity, experimental allergic encephalomyelitis (EAE), a demyelinating disease is induced following subcutaneous administration of proteolipid protein (PLP). In the EAE model system, studies have shown that blocking B7-1 interactions decreases the incidence of disease, while similarly blocking B7-2 interactions favors increased disease severity (Kuchroo et al., 1995; Racke et al., 1995). In one model of EAE, treatment of PLP-immunized mice with anti-B7-1 mAbs was associated with less severe EAE and the production of T cell clones that secreted relatively large amounts of IL-4 (Ranger et al., 1996). These findings indicate that costimulation through B7-2 interactions may promote a protective Th2 immune response, and furthermore, suggest that B7-1 and B7-2 could function distinctly through their individual interactions with CD28 and/or CTLA-4, thereby promoting

different types of cytokine responses and subsequent disease outcomes. Similar differential effects of blocking B7-1 versus B7-2 have been observed in other EAE models (Racke et al., 1995). In another model, the non-obese diabetec (NOD) mouse that develops autoimmune diabetes, blocking B7-2 favors protection, while inhibition of B7-1 enhances disease severity (Lenschow et al., 1995). In these different systems, both Th1 and Th2 components are present throughout the course of the autoimmune disease.

Recently, Han et al. (1995) examined the role of B7-2 during a T dependent systemic immune response to the protein antigen, chicken γ-globulin. Treatment with blocking B7-2 mAbs at the time of immunization inhibited GC formation and reduced serum immunoglobulin levels to 50% of controls. Interestingly, delaying administration of blocking B7-2 mAbs to days 6-10 following immunization did not block GC formation, but did impair immunoglobulin hypermutation and B cell memory development. To further reveal the functional roles of B7-1 and B7-2 and eliminate the possibility that antibodies are incompletely inhibiting the cell surface molecules, Wu et al. (1997) developed single and double B7-1 and B7-2 knockouts. During the immune response to the T-dependent protein antigen, TNP-KLH, administered by various routes and in the presence or absence of adjuvant, mice lacking both B7-1 and B7-2 failed to exhibit serum immunoglobulin class switching or form GCs. In contrast, either B7-1 or B7-2 deficient mice immunized with TNP-KLH and complete Freund's adjuvant (CFA) demonstrated marked increases in antigen-specific IgG. In the absence of CFA, B7-2 deficient mice immunized intravenously with TNP-KLH were unable to form GCs or exhibit serum immunoglobulin isotype class switching while B7-1 deficient mice

exhibited elevations in serum IgG and GC formation similar to controls. These results highlight the importance of adjuvants in determining the differential effects of B7-1 versus B7-2 *in vivo*.

While B7-1 and B7-2 may have redundant roles in certain types of responses, B7-2, but not B7-1, may be required during systemic responses in the absence of adjuvant. Concurrently with these studies, my initial thesis work focused on the role of B7-1 and B7-2 during the initiation of an *in vivo* type 2 mucosal immune response to the nematode parasite, *H. polygyrus*. In contrast to the autoimmune disease models and the T dependent immune responses to protein antigens, live pathogens often elicit strong, highly polarized Th1 or Th2 responses, and when our initial paper was published, few studies had examined the role of B7-1 versus B7-2 in providing costimulation for T cell effector function during immune responses to infectious pathogens. Our studies were initially carried out using antibody intervention experiments, and later, complementary studies were completed in knockout mice. Given the different conclusions about the relative roles of B7-1 versus B7-2 in mediating costimulatory interactions with CD28/CTLA-4 and subsequent T cell differentiation during the type 2 immune response, the parasite model system seemed appropriate for examining the individual function of these costimulatory molecules *in vivo*.

In this project, the role of B7-1 and B7-2 costimulatory signals in the development of effector cell populations associated with the *in vivo* Th2 mucosal response to *H. polygyrus* was examined. This response, which resembles that associated with allergy, includes local tissue edema, increases in mucosal mastocytosis and blood eosinophilia;

elevations in TCRα/β^+, $CD4^+$ T cell derived IL-4 production; T-dependent and -independent elevations in IL-3, IL-5 and IL-9 mRNA; and B cell proliferation and differentiation that results in marked increases in serum IgE and IgG1 and mesenteric lymph node (MLN) GC (GC) formation (Svetic et al., 1993; Gause et al., 1995). IL-3, IL-5, and IL-9 are elevated within 6 hours after oral inoculation with *H. polygyrus* larvae while IL-4, primarily derived from $CD4^+$, TCR-$\alpha\beta^+$ T cells, is not elevated until 4-6 days (Svetic et al., 1993). By day 14, pronounced elevations in serum IgE and IgG1, and in GC formation are evident. In this chronic primary response to a murine pathogen, the worms are not expulsed but continue to stimulate a Th2 response for several weeks, although the response is not as pronounced as that detected during the first 8-10 days (Finkelman et al., 1997).

Blocking B7 ligand interactions with the murine fusion protein CTLA-4Ig, which inhibits both CD28 and CTLA-4 interactions with its ligands, has been shown to inhibit the development of IL-4-producing T cells and the associated Th2 immune response to a number of infectious pathogens, including the nematode parasite *H. polygyrus* (Lu et al., 1994) and also the intracellular protozoan, *L. major* (Corry et al., 1994). In the first part of this project, our antibody intervention studies demonstrated that either B7-1 or B7-2 ligand interactions can provide the costimulatory signal necessary for T cell differentiation to effector cell function during a Th2 immune response.

After our initial studies on the role of B7-1 versus B7-2 were published, several other studies examined the role of B7-1 versus B7-2 in providing costimulatory signals during immune responses to infectious pathogens. These results of these studies

demonstrate that the type 2 immune responses to both *Schistosoma mansoni* (Subramanian et al., 1997) and *L. major* (Brown et al., 1997) are inhibited following anti-B7-2 mAb treatment. Taken together with our studies, these data suggest that B7-2 requirements during a Th2 immune response may differ with different infectious pathogens. However, the use of blocking anti-B7-1 and B7-2 antibodies is associated with potential difficulties in interpretation including incomplete blocking, which can be particularly important at later stages of the immune response when blocking antibodies may induce their own response resulting in the production of endogenous antibodies that neutralize the effects of the exogenously administered blocking antibodies (Gause and Finkelman et al, personal communication).

It is also possible that anti-B7-1 and anti-B7-2 mAbs may be stimulatory since differences were observed following *in vivo* administration of whole antibody versus Fab fragments (Miller et al., 1995), presumably because the Fab fragments cannot crosslink and trigger signal transduction. However, it is also possible that the short half-life of Fab fragments or immune responses to the Fab fragments, which are often administered at high doses, also contribute to differences from whole antibody. As mentioned earlier, the recent development of mouse strains lacking B7 molecules has provided a useful alternative model for comparison with the *in vivo* effects of blocking antibodies. Studies with these knockout mice have indicated that, when immunized with the protein antigen, TNP-KLH, plus CFA, either B7-1 or B7-2 can support the development of GC formation and serum IgG responses, while in the absence of adjuvant, B7-2 interactions are required (Borriello et al., 1997).

To directly examine whether B7-2 is required for the development of the Th2 response and to examine the role of B7-2 at later stages of the chronic response, B7-2KO mice were immunized with the nematode parasite, *H. polygyrus*. Our studies show that T helper effector cell function and the associated Th2 immune response are not initially inhibited in B7-2-deficient mice, consistent with our studies with blocking antibodies. However, at later stages of the immune response, T cell cytokine elevations and serum IgE elevations are inhibited, although other components of the Th2 response, including serum IgG1 elevations and GC formation, are intact. These findings demonstrate that B7-2 differentially regulates effector cell function during a Th2 response, both with regards to the stage of the response and the particular effector cell functions involved. In addition, these data may provide one explanation for the variability in B7-2 dependency that has been observed following infection with different pathogens and using different immunization regimens.

E. CD28 and CTLA-4

The B7 ligands, CD28 and CTLA-4, are expressed primarily on T cells and show only 27-31% homology at the amino acid level within the same species, although rigid conservation is observed within a 16 amino acid stretch of the complementarity determining region 3 (CDR3)-like domain that includes the MYPPPY motif (Linsley et al., 1994; June et al., 1994). CD28 is constitutively expressed on T cells, whereas CTLA-4 is induced following T cell activation (Linsley et al., 1992). CTLA-4 is not detectable

on resting T cells, but is rapidly upregulated after T cell activation (Lindsten et al., 1993). In contrast to the B7 molecules, both CD28 and CTLA-4 exhibit highly conserved cytoplasmic domains between species, which suggests an important role in T cell signaling for these molecules. In fact, there is 100% amino acid conservation between the cytoplasmic domain of human and mouse CTLA-4. A current area of active research is focused on the downstream signaling pathways of CD28 and CTLA-4 and how these pathways relate to T cell receptor signaling.

Although our understanding of the multiple CD28 signaling pathways is incomplete, several recent studies have begun to clarify the intricacies of its molecular interactions. Signaling through CD28 can be roughly divided into four chronological events: early signals, intermediate events, transcriptional nuclear events and subsequent cytoplasmic translational events (Ward, 1996; Reif and Cantrell, 1998). Crosslinking of CD28 stimulates early signaling events via associations with its cytoplasmic tail. The CD28 cytoplasmic tail consists of four highly conserved tyrosine residues, each of which are potential sites for tyrosine phosphorylation (June et al., 1994). One tyrosine is situated in a YMNM motif that is recognized by the SH2 domain of phosphatidylinositol 3-kinase (PI 3-kinase) (Wang et al., 1995), GRB-2 (growth factor receptor bound protein) (Schneider et al., 1995), and T cell-specific protein-tyrosine kinase (August et al., 1994) (Rudd, 1996).

Based on evidence from studies using purified resting T cells, T cell lines, stimulation of CD28 with monoclonal antibodies has been shown to activate phospholipase C, $p21^{ras}$, inositol $(1,4,5)P_3$, PI 3-kinase, sphingomyelinase/ceramide, 5-

lipooxygenase and induce calcium elevations (Ward, 1996). Each of these pathways may contribute to early signal events; however relatively little is known about the level of interaction among the multiple signaling pathways and their individual roles in CD28 signaling. Given the complexity of possible signaling events following CD28 ligation, it is conceivable that a variety of outcomes may occur depending on factors such as the strength of CD28 signaling and the overall level of T cell activation.

Following early signaling events, a series of intermediate proteins link upstream signals to downstream nuclear events. Recent studies have shown that the rho family, which includes RhoA, RhoB, RhoC, Rac 1, Rac 2, Cdc42Hs, RhoG and TC10, are critical intermediaries for the downstream regulation of cytokine gene expression, cell survival, clonal expansion and cytoskeletal conformation (Reif and Cantrell, 1998). Rho family members themselves are subjected to a level of upstream regulation by Rho family GTPases, which function primarily as early regulators of the phosphorylation state of cell signaling molecules. Studies have demonstrated marked upregulation of tyrosine-phosphorylated Vav1 (a Rho family member that acts a guanine nucleotide exchange factor) following antigen receptor binding in T cells, and complex formation between Vav1 and SLP-76 occur following both TCR and CD28 receptor ligation (Wange and Samelson, 1996; Wu et al., 1997). In addition to common signaling pathways activated by TCR and CD28 receptor ligation, there are also distinct activation pathways unique to CD28. Interaction of B7-1 and B7-2 with CD28 has been shown to stimulate the tyrosine phosphorylation of $p62^{dok}$ (a 62kDa adapter protein), which is not induced by TCR ligation (Nunes et al., 1996; Gause et al., 1997b; Yamanashi and Baltimore, 1997).

Activated $p62^{dok}$ associates with proteins involved in the downstream Rho and Rac signaling pathways, which regulate a variety of vital cellular processes.

Th early and intermediate signaling events direct the subsequent nuclear events. Several key transcriptional activators have been identified following CD28 signaling. In particular, AP-1, NFκB, NFAT, and Oct-1 are critical activators of the IL-2 promoter complex (Ward, 1996). Less is known about post-transcriptional regulatory pathways. It is likely that there is an additional level of control at the translational level involving stabilization of mRNA which permits prolonged gene expression (Lindsten et al., 1989). Our understanding of the downstream events following Ag-specific TCR ligation and costimulatory CD28 interactions are incomplete. Further definition of these events will provide insight into the role(s) that these cell surface molecules play during immune responses.

F. Functional studies of CD28 and CTLA-4

The B7 ligands, CD28 and CTLA-4, share considerable homology and ligand binding specificity (June et al., 1990). Although initial studies suggested that both molecules mediated T cell costimulation (Linsley et al., 1992), more recent evidence suggests that CTLA-4 may instead provide a negative signal. Cross-linking of CTLA-4 on resting T cells can inhibit IL-2 production and cell proliferation induced by a combination of anti-CD3 and anti-CD28 mAbs (Krummel and Allison, 1996). Furthermore CTLA-4KO mice develop severe lymphoproliferation and associated tissue destruction of multiple organs (Tivol et al., 1995; Waterhouse et al., 1995). In contrast,

CD28KO mice exhibit a relatively normal phenotype; however, in some cases the immune response is markedly impaired. In CD28KO mice, basal Ig levels are only 20% of those observed in normal mice (Shahinian et al., 1993). T cells isolated from CD28KO mice exhibit impaired responses to alloantigens, nominal antigen, and anti-CD3 mAbs (Green et al., 1994) and GC formation is blocked in CD28-deficient mice in response to immunization with the hapten-carrier protein, nitrophenyl-acetyl chicken γ-globulin (NP-CG) (Ferguson et al., 1996). However, T cell cytotoxicity is intact in CD28KO mice and can mediate a host protective delayed-type hypersensitivity response following LCMV infection (Shahinian et al., 1993). Although these studies suggest major defects in the immune response of CD28-deficient mice, other studies involving infectious agents suggest a more intact response. In this project, the role of CD28 and CTLA-4 were examined using these knockout mice.

To complement our functional studies of the B7 molecules on APCs, the individual roles of the T cell surface CD28 and CTLA-4 were examined with antibody intervention studies and experiments in gene knockout mice. We have previously investigated the role of B7 ligands during *in vivo* T cell differentiation to IL-4 producing cells using two different type 2 cytokine immune response models: 1) the mucosal immune response that follows oral inoculation with infectious larvae of *H. polygyrus*; and 2) the systemic immune response to an intraveneously injected immunogenic anti-IgD Abs. In these two systems, $CD4^+$ $TCR\alpha\beta^+$ T cells rapidly differentiate to produce IL-4, but differ in the primary site of lymphoid activation (mucosal versus systemic, respectively) and in the APCs involved (Svetic et al., 1991; Svetic et al., 1993). In both

of these systems, our studies have shown that CTLA-4Ig administration at the initiation of the primary immune response blocks T cell activation, which leads to IL-4 production, and also inhibits other T-dependent effector cell functions, including B cell activation and differentiation to Ab secretion (Lu et al., 1994; Lu et al., 1995).

In this thesis I have further demonstrated that in CD28 deficient mice, the type 2 immune response leading to T cell IL-4 production and elevations in serum IgE and IgG1 levels is completely blocked in mice immunized with goat anti-IgD (GaMδ) Abs, but is essentially intact in mice infected with *H. polygyrus*. Studies of the intact response *to H. polygyrus* included the first demonstration that T cells can differentiate to IL-4 production in the absence of CD28. Thus, one focus of this dissertation is to define the functional role of CD28 during an *in vivo* type 2 immune response and to address the possibility of an alternate costimulatory pathway in the absence of CD28 costimulation.

G. Germinal Center Development

The GC is an *in vivo* site of B cell activation, differentiation, and memory cell development (MacLennan, 1994). GCs form in the T cell rich regions of secondary lymphoid tissue within a follicle of highly specialized antigen presenting cells called follicular dendritic cells (FDC). As antigen is delivered through the lymph from the site of infection to the local secondary lymphoid tissues, such as the spleen, MLN, and Peyer's Patches (PP), professional antigen presenting cells (B cells, dendritic cells, macrophages) process and present the antigen in conjunction with MHC class II molecules. Antigen-specific naïve $CD4^+$ T helper cells initially interact with APCs in the

T cell rich regions of the lymphoid tissues (Kelsoe, 1995). These initial costimulatory interactions are required to activate B cells, which then migrate into the follicles and clonally expand to form GCs. Thus, GC formation is an excellent gauge of T helper cell effector function required for B cell activation and differentiation.

Following initial costimulatory interactions with Th cells in the T cell rich regions of the lymphoid tissues, newly activated B cells migrate into the GC where rapid cell division and clonal expansion occur along with somatic hypermutation of the Ig V-regions (Kelsoe, 1995). As the GC expands, B cells interact with Th cells and with FDCs, which have immune complexes of antigen on their long, projection-like cell surface. The B cells with the highest affinity antigen receptors bind with the tightest affinity to the antigen complexed on the surface of APCs and receive cell survival signals (Kelsoe, 1995). However, the majority of B cells do not bind well to the FDCs, die by apoptosis and are eliminated by tingible body macrophages (Kelsoe, 1995). The GC environment is highly dynamic and evolutionarily designed to select for the B cells that secrete the highest affinity antibodies and which subsequently return to the site of infection. Moreover, although memory B cells develop within the GC, the molecular interactions required for their development are not well-understood.

H. Costimulatory Requirements for Memory T cell Development:

The induction of long-lived antigen-specific memory cells is critical to the development of an effective vaccine. For instance, a vaccine candidate that elicits a strong effector response without the development of a memory response will be

ineffective as an immune defense during subsequent exposure to pathogens (or antigen). Memory cells provide the potential for a more rapid control of infection during a challenge immune response. Memory T cells live longer than effector T cells, differentiate rapidly to effector cells, and are generally committed to a specific cytokine pattern that was selected during the primary response (Swain, 1994; Bruno et al., 1995). Although some studies have suggested that memory T cells develop from effector T cells (Swain, 1994; Bruno et al., 1995), the differentiation pathways required for the induction of memory T cells are poorly understood.

Previous studies in our laboratory have demonstrated that B7 ligand interactions are essential for the development of effector T cells from naïve (Lu et al., 1995; Lu et al., 1994), but not memory T cells (Gause et al., 1996), since CTLA-4Ig administration at the time of challenge did not effect worm expulsion during the challenge immune response to *H. polygyrus*. However, few studies have examined the role of costimulatory molecules in the induction of T cell memory during the primary immune response. In the *H. polygyrus* system, the primary immune response is associated with chronic infection and following treatment with an anti-helminthic drug, the subsequent challenge immune response is host protective. Thus, the development of a memory T cell response is clearly distinguishable from an effector response based on several parameters including host protective worm expulsion. One focus of the dissertation was to determine whether blocking B7 ligand interactions in the primary immune response inhibits the development of memory T cells or induces T cell tolerance. To address this question, an experiment was designed where CTLA-4Ig was administered at the initiation of the primary immune

response to *H. polygyrus,* the animals were treated with an anti-helminthic drug, and subsequently challenged with *H. polygyrus*. The mice were challenged after the detectable primary immune response had diminished and assayed for T and B cell surface activation parameters as detected by flow cytometry, Th2 cytokine production, serum Ig elevations, GC formation, blood eosinophilia, and intestinal mastocytosis. We are also currently examining the role of alternative T cell costimulatory molecules, such as heat-stable antigen (HSA) in the generation of memory T cells, since our findings suggest that blocking B7 ligand interactions during the primary response does not block the host protective challenge response.

Recent studies of mice deficient for HSA and/or CD28 suggest that B7 ligand interactions are required for the development of effector cytotoxic T cells during a viral immune response, while either HSA or B7 ligand interactions can provide the required costimulatory signaling for memory cytotoxic T cell induction (Liu et al., 1997). It is suggested that a strong stimulus provided by B7 is required for effector T cell development, while a weaker costimulatory signal, which could be provided by HSA, can be utilized for the induction of memory T cells. It is important to examine whether this finding occurs in other systems such as *H. polygyrus*.

HSA is a T cell costimulatory molecule expressed on the surface of hematopoietic and neuronal cellular types. In several model systems, HSA in conjunction with B7 have been shown to be important in activating naïve T cells. The recent development of HSA deficient and HSA/CD28 double knockout mice will allow us to address the role of these molecules in the generation of memory T cells. These types of studies are critical for

determining the specific cellular interactions required for memory cell development. Future immunotherapies will be designed based on maximizing the memory cell response, thereby fostering rapid antigen clearance.

I. *Heligosomoides polygyrus*: A model system for type 2 mucosal immune responses

H. polygyrus was isolated in 1845 by F. Dujardin from different rodent species in France. A century later, G. M. Spurlock isolated this parasite from wild *Mus musculus* in Cailifornia and developed an experimental mouse model (Behnke et al., 1991). The infective L3 larvae were maintained in a stable condition and thus, strain variation due to numerous passages was avoided. The laboratory strain that is most frequently used was isolated and established in a mouse model in 1950 by F. A. Ehrenford (Monroy and Enriquez, 1992). There is an extensive literature using *H. polygyrus* to examine multiple aspects of gastrointestinal disease. During the primary immune response to *H. polygyrus*, the mice develop a chronic infection characterized by the rapid development of a type 2 mucosal immune response. If an anti-helminthic drug, pyrantel pamoate, is administered during the primary response to *H. polygyrus*, the worms are rapidly eliminated from the gut. Upon subsequent challenge with *H. polygyrus*, a host protective memory response is mounted and the worms are expelled from the gut. This model is particularly useful for studying both chronic primary and host protective memory responses to an infectious pathogen. The studies presented in this dissertation will focus on the role of B7:CD28/CTLA-4 interactions in the development of the type 2 immune response to *H. polygyrus*.

H. polygyrus is a natural parasite of mice and therefore, a well-suited model for the *in vivo* study of host-parasite interactions that influence the development of the immune response. Animals are orally inoculated with free-living, ensheathed L3 larvae. The ingested larvae migrate to the proximal third of the small intestine and encyst within the intestinal wall where maturity into adult worms occurs. By day 8, local tissue edema, elevations in intestinal mastocytosis, blood eosinophilia, increases in Th2 cytokine production and B cell activation rapidly develop following infection. Increases in these Th2 immune response parameters can be detected by assays developed in our laboratory and are an important readout of the host response to the parasite.

To further define the function of molecules that mediate cell:cell interactions required for effector T cell development, this project has incorporated antibody intervention studies with complementary experiments in gene knockout mice. In these studies with various treatments or genetic manipulations, the *H. polygyrus* immune response is examined repeatedly with assays designed to detect the *in vivo* type 2 immune response. The consistency of our assays has been critical in our ability to functionally define important activation molecules on the T helper and antigen presenting cell, as well as to measure the effectiveness of potential immunotherapies designed to alter the immune response.

J. Specific Goals of the Project

This dissertation is focused on the regulation of the development of a type 2 *in vivo* mucosal immune response by targeting B7:CD28/CTLA-4 interactions. The initial

studies focused on whether B7-1 or B7-2 can provide the required CD28/CTLA-4 ligand interactions for the development of the primary immune response. Later studies further defined the functional role of CTLA-4 at the initiation of the immune response to *H. polygyrus*. Finally, the costimulatory requirements for the generation of memory T cells from naïve T helper cells during the immune response to *H. polygyrus* were examined.

II. Materials and Methods

Animals. Female BALB/c mice were purchased from the Small Animal Division of the National Institutes of Health (Bethesda, MD) and were used at 8 to 12 weeks of age for all experiments involving wild-type mice. For the experiments comparing *H. polygyrus* versus anti-IgD immunization in CD28WT and CD28KO mice, female C57BL/6 (H-2^b) mice (aged 6-8 weeks) were purchased from the Jackson Laboratory (Bar Harbor, ME). CD28KO mice were further back-crossed to C57BL/6 genetic background (C57BL/6 x CD28KO) F5. For the experiments involving CD28WT and CD28KO mice administered CTLA-4Ig, female BALB/c mice (aged 6-8 weeks) were acquired from Tak Mak (University of Toronto, Canada). BALB/c B7-2WT and B7-2KO mice (aged 6-8 weeks) were used in our studies and given to us in collaboration with Arlene Sharpe (Harvard University, Boston, MA). The experiments reported herein were conducted according to the principles set forth in the "Guide for the Care and Use of Laboratory Animals", Institute of Animal Resources, National Research Council, Department of Health, Education and Welfare.

Antibodies. Abs and reagents used for the *in vivo* cytokine intervention experiments include rat anti-mouse B7-2 (GL1) (Hathcock et al., 1994), hamster anti-mouse B7-1 (16-10A1) (Xu et al., 1994), hamster anti-CTLA-4 (4F10), rat anti-CD28 (37N), control isotype-matched rat IgG2a (GL117) and control normal Syrian hamster IgG. Murine

CTLA-4Ig and the control fusion protein L6 were provided by Dr. Peter Linsley (Bristol-Myers Squibb, Seattle, WA). For FACS analysis, the biotinylated anti-MHC II (Ia^d, MKD6) (Kappler et al., 1981), FITC conjugated anti-CD4 (GK1.5) (Dialynas et al., 1983), anti-TCRαβ (H57-597, Pharmingen, San Diego, CA), biotinylated anti-IL-2Rα (Pharmingen), cychrome anti-B220 (6B2) (Morse et al., 1982) Abs and anti-FcγRII (24G2) (Unkeless, 1979) were used. As a secondary conjugate for the biotinylated Abs, strepavidin-phycoerythrin (Becton Dickinson, Franklin Lakes, NJ) was used. For the ELISPOT assay, a pair of anti-IL-4 Abs (BVD4.1D11.2 and BVD6.24G2.3) that bind to different epitopes on the IL-4 molecule were used (Chatelain et al., 1992).

Chemicals and reagents. The ELISPOT assay and FACs analysis require the following reagents: RPMI-1640 and HBSS media (BioWhittaker, Walkersville, MD); fetal bovine serum (HyClone, Logan,UT); Tween 20, sodium azide, 2-amino-2-methyl-1-propanol, sodium azide, 5-bromo-4-chloro-3-indoyl-phosphate (BCIP) and bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO); low-melt seaplaque agarose (FMC BioProducts, Rockland, ME); strepavidin-alkaline phosphatase (Jackson Immuno Research Lab, West Grove, PA); immunolon II plates (Dynatech Laboratories, Chantilly, VA); and RNAzol (Cinna-biotech, Friendswood, TX). The following reagents were used for the RT-PCR reaction: Taq polymerase (Promega, Madison, WI), superscript RT (Life Technologies, Gaithersburg, MD), gamma ATP-^{32}P (ICN, Irvine , CA), T4 kinase and dNTPs (Pharmacia Biotech, Piscataway, NJ).

Parasitologic Parameters. Infective, ensheated, third-stage larvae of *H. polygyrus* (specimens on file at the U.S. National Parasite Collection, U.S. National Museum Helminthologic Collection no. 81930, Beltsville, MD) were propagated and stored at 4°C until used. Mice were inoculated orally with 200 larvae using a ball-tipped feeding tube (Urban et al., 1991). The larvae were propagated on vermiculite-fecal cultures, separated from culture using a Bearmann apparatus, washed in saline, counted and stored at 4°C until used for inoculation.

Immunization of mice with anti-B7-1 and anti-B7-2 mAbs. After inoculation with *H. polygyrus*, mice were injected i.v. via the tail vein on either day 0 or 4 with 100 μg of anti-B7-1 Ab and/or 100 μg of anti-B7-2 Ab and analyzed. As a control, *H. polygyrus*-inoculated mice were similarly administered 100 μg of an isotype-matched rat IgG or normal hamster IgG.

Immunization of mice with CTLA-4Ig. Mice were injected i.v into the tail vein with 200 μg of murine CTLA-4Ig or the control fusion protein, L6, at days 0 and 1 following immunization.

Immunization of mice with anti-IgD Abs. Mice were injected i.v. into the tail vein with 800 μg of affinity purified goat anti-IgD Abs in 100 ml of 0.15M NaCl (Svetic et al., 1991; Finkelman et al., 1987).

Immunization with anti-CD28 and anti-CTLA-4 mAbs. *H. polygyrus*-inoculated mice were administered 300 μg of anti-CD28 mAbs on days 0 and 3 and/or 450 μg and 200 μg of anti-CTLA-4 mAbs on days 0 and 3, respectively. As a control, *H. polygyrus*-inoculated mice were similarly administered 300 μg of isotype-matched rat IgG on days 0 and 3 and/or 450 μg and 200 μg of normal hamster IgG on days 0 and 3.

Cell labeling and analysis. For cell sorting and FACs analysis, MLN cells were washed with HBSS + 0.1% BSA + 0.1% sodium azide, filtered through nylon mesh (Martin Supply Co., Baltimore, MD), centrifuged at 1200 x G in a refrigerated Sorvall RC-3B centrifuge and resuspended in the HBSS media. After counting the cells with a Coulter counter (Coulter electronics, Hialeah, FL), 1 x 10^6 MLN cells were simultaneously stained with appropriate dilutions of FITC-conjugated anti-MHC class II Ab and CY5 conjugated anti-B220 Ab to assess B cell activation and with FITC-conjugated anti-CD4 and CY5-conjugated anti-IL-2R to assess T cell activation. Dual-color flow cytrometric analysis was accomplished with an Epics ELITE flow cytometer (Coulter Electronics, Hialeah, FL). An argon laser was used as the excitation source for both FITC and phycoerythrin.

Immunohistological analysis. The procedure used for immunohistologic staining was that described previously with some modifications (Han et al., 1995). Briefly, mesenteric

lymph nodes (MLN) were collected from each treatment group on day 8 after *H. polygyrus* inoculation and frozen in liquid nitrogen in 15 ml canisters containing 4 ml of frozen tap water. Sections were cut serially at 8μm (approximately one-cell thick) with a cryostat microtome, mounted onto slides and stored at -70°C. Before staining, frozen slides were warmed to room temperature, fixed in cold acetone for 10 minutes, and treated with a 0.03% H_2O_2/PBS solution for 5 minutes to eliminate endogenous peroxidase activity. $CD4^+$ T cells were stained with biotinylated anti-CD4 mAb (L3T4) followed by strepavidin-alkaline phosphatase (Zymed laboratories, Inc., San Francisco, CA). After washing, GC B cells were stained with horseradish peroxidase conjugated to peanut agglutinin (PNA) (ICN Biomedicals, Aurora, OH). The phosphatase/Fast Blue BB (Sigma Chemical Co.) and then the peroxidase was developed with 3-amino-9-ethylcarbazole (Sigma Chemical Co.). MLN sections were collected from each of the treatment groups and were analyzed for the staining patterns of $CD4^+$ T cells and GC B cells. GC formation was quantified volumetrically by staining three planes per MLN tissue with PNA to detect GCs (five animals per treatment group) and the ratio of PNA^+ GC B cells to total lymphoid tissue at three planes per MLN tissue were calculated with photographs of each section. All photographs of the tissue sections shown in the figures were taken at the same magnification (approx. X125).

Quantitation of serum immunoglobulins. Serum IgG1 and serum IgG2a levels were quantitated by radial immunodiffusion with standards and materials purchased from

Hazelton Laboratories (Rockville, MD). Serum IgE levels were assayed by a micro-ELISA developed in our laboratory.

Eosinophil counts. Eosinophils were counted from fresh blood samples with the Unopette test (Becton Dickinson, Rutherford, N.J.).

Evaluation of mucosal mast cell (MMC) number. Tissue samples from 8- to 10-cm mid-jejunal segments from individual mice were prepared by the Swiss roll technique. This technique involves slicing the intestine open, removing the contents, and arranging the gut in a straight line. With a toothpick held perpendicular to the intestine, the gut is rolled into a circle, removed from the toothpick with a tweezer, and placed into Carnoy's fixative. The tissue is later paraffin embedded and processed for staining with Alcian blue and safranin. After a random selection of the first microscopic field (at a magnification of X397), a total of 50 neighboring fields of a given section of rolled jejunum were scanned for MMC. MMC were identified in both the villi and crypts by their distinct intracellular staining with Alcian blue (Madden et al, 1991). All samples were evaluated blindly.

ELISPOT. The frequency of IL-4 producing cells was determined by an ELISPOT assay. Briefly, individual wells of Immulon II polystyrene 96-well flat-bottom plates were precoated with the anti-cytokine capture Abs (BVD4.1D11.2 for IL-4) at a concentration of 10μg/ml and incubated at 4°C overnight. After three PBS/Tween 20

(0.05%) washes and three PBS washes, plates were blocked with RPMI 1640 and washes and 5% fetal bovine serum for 1 hour at 37°C. Single cell suspensions (0.1 ml) adjusted to a concentration of 5 x 10^6 cells/ml, were added to the coated plates in serial five-fold dilutions and incubated for 3 hours at 37°C. Plates were then washed three times with PBS and three times with PBS/Tween 20 after which a biotinylated anti-cytokine antibody (BVD6.24G2.3 for IL-4) was added to the wells at a concentration of 4 μg/ml. After incubation for 1 hour at 37°C, the plates were washed three times with PBS, then three times with PBS/Tween 20, after which strepavidin-alkaline phosphatase (Jackson Immuno-Research, West Grove, PA), diluted 1/2000 in PBS/Tween 20 and 5% fetal bovine serum, was added to the wells, and then the plates were incubated for an additional hour at 37°C. Plates were then washed five times in PBS and 1 μg/ml of 5-bromo-4-chloro-3-indoyl phosphate diluted in 0.1M 2-amino-2-methyl-1-propanol, with 0.6% low melt agarose was added to individual wells. After overnight incubation in a humid chamber, the number of blue spots in individual wells (where each spot represents a single cytokine-secreting cell) were enumerated by examining wells under a dissecting microscope. Using an inverted microscope, the number of spots were counted per well with a cell counter. For the ELISPOT assay, MLN suspensions from the animals were plated individually and duplicated, so that the counts per animal were the average of two wells and the standard error could be determined between five animals per treatment group. The count per well represents the number of IL-4 secreting cells per five million MLN lymphocytes.

Purification of RNA. Immediately following tissue collection, tissues were homogenized in 1.5 ml of RNAzol (Cinna/Biotecs) at a concentration of approximately 50 mg of tissue/ml. The homogenized tissues in propylene tubes were frozen in liquid nitrogen and stored at -70°C until purification. To extract the RNA, the samples were warmed at 37°C for 5 minutes and transferred into 2 ml tubes. 0.15 ml of chloroform was added to the samples and the samples were vortexed for 15-20 seconds. The samples were centrifuged at 14,000 x g in a refrigerated Eppendorf centrifuge 4515C (Brinkman Instruments, Westbury, NY) for 15 minutes. The aqueous phase was pipetted into another tube and an equal volume of isopropanol was added. The samples were mixed and incubated at -20°C for 1 hour or overnight. After incubation, the samples were centrifuged for 15 minutes at 14,000 x g in the refrigerated Eppendorf centrifuge. The supernatant was discarded, the RNA pellet was washed twice with 70% ethanol and dried in a vacuum by a Speed Vac concentrator (Savant, Hicksville, NY). To dissolve the pellet, 50 µl of RNA-ase free water was added to the samples and stored at -70°C. To quantify the RNA, 1 µl of sample was diluted in 59 µl of distilled water. The concentration and OD 260/280 ratio were determined for each sample.

Reverse transcription. To start the reverse transcription reaction, 1.8 µg of RNA was diluted into a volume of 11.8 µl of RNA-ase free water and heated to 37°C for five minutes and then, placed on ice. To this solution, 13.2 µl of a well-mixed master mix was added to each sample for a final volume of 25 µl. The master mix consists of: 2.5 µl

of a 10 mM mix of the four deoxynucleotide triphosphates; 5 μl of 5X RT buffer (260 mM Tris-HCL, 375 mM KCL, 15 mM $MgCl_2$); 2 μl of dithiothreitol (0.1 M); 2 μl of random hexamers (0.5U/μl); 0.5 μl of RNAsin (40U/μl); and 1.2 μl of reverse transcriptase (200U/μl). The samples were incubated for 37°C for 60 min., denatured at 90°C for 5 min., and cooled on ice for 5 min. The samples were stored at -20°C.

Polymerase Chain Reaction (PCR).

Primers and Probes. As shown in Table I, the primers and probe sequences are listed. Using PC/GENE (IntelliGenetics, Mountain View, CA), each cytokine cDNA sequence was downloaded and with the program, individual primer sequences were designed. Primers characteristically amplify a 200-400 base pairs (bp) region and are 18-25 bp with 50% GC content. The probes are designed similarly to the primers; however, the probes target sequences in the middle of the PCR amplified DNA product on the Southern blot.

PCR preparation. A PCR master mix was made for the PCR reaction which contains the following: 4.0 μl of dNTPs (2.5 mM), 5.0 μl of 10X Taq polymerase buffer, 3.0 μl 25 mM $MgCl_2$, 0.2 μl of Taq polymerase (5 U/μl), 2.0 μl of sense oligo primer (0.2 μg/μl),

Table I. Sense and anti-sense primer and probe sequences for the amplification of cytokine cDNA during the PCR and detection of amplified DNA on Southern blot (Svetic et al., 1993; Greenwald, unpublished).

Cytokine	Primer and Probe Sequences		# of PCR Cycles
IL-3	Sense	GAG TCA AAT CCA GAA CAT GCC	28
	Anti-sense	TCC ACT TCA AGC TCT ACA G	
	Probe	CTC CCC AGG ATG CTC ACC TTC	
IL-4	Sense	CTC AGT ACT ACG AGT AAT CCA	23
	Anti-sense	GAA TGT ACC AGG AGC CAT ATC	
	Probe	AGG GCT TCC AAG GTG CTT CGC ATA TTT	
IL-5	Sense	GAC AAG CAA TGA GAG ACG ATG AGG	26
	Anti-sense	GAA CTC TTG CAG GTA ATC CAG G	
	Probe	GGG GGT ACT GTG GAA ATG CTA T	
IL-9	Sense	TGA TGA TTG TAC CAC ACC GTG	28
	Anti-sense	CCT TTG CAT CTC TGT CTT CTG G	
	Probe	GCC TGT TTT CCA TCG GGT GAA A	
IL-10	Sense	CGG GAA GAC AAT AAC TG	20
	Anti-sense	CAT TTC CGA TAA GGC TTG G	
	Probe	GGA CTG CCT TCA GCC AGG TGA AGA CTT TCT TT	
IL-13	Sense	CTC CCT CTG ACC CTT AAG GAG	28
	Anti-sense	GAA GGG GCC GTG GCG AAA CAG	

	Probe	TCC AAT TGC AAT GCC ATC TAC	
IFN-γ	Sense	ACC GCT ACA CAC TGC ATC TTG G	20
	Anti-sense	GAC TTC AAA GAG TCT GAG G	
	Probe	GGA GGA ACT GGC AAA AGG A	
HPRT	Sense	GTT GGA TAC AGG CCA GAC TTT GTT G	12
	Anti-sense	GAT TCA ACT ACT TGC GCT CAT CTT AGG	
	Probe	GTT GTT GGA TAT GCC CTT GAC	

2.0 µl of anti-sense primer (0.2 µg/µl), and 31.3 µl of purified water. To prepare the master mix, the number of samples was multiplied by each volume of the master mix components. Before starting the PCR reaction, 2.5 µl of cDNA from the reverse transcription reaction was transferred into a 0.5 ml PCR tube containing 47.5 µl of the master mix. The tubes were briefly centrifuged and one drop of mineral oil was added to each tube.

PCR program conditions. After an initial incubation at 95°C, the PCR reaction was programmed for each cycle in a DNA thermal cycler (Perkin Elmer, Norwalk, CT) as follows: denaturation at 94°C for 45 sec.; annealing at 53°C for 1 min.; and extension at 72°C for 2 min. Following completion of the cycles, an additional extension period at 72°C of 7 min. was carried out and the samples were stored at 4°C. For each cytokine, the optimum number of cycles (i.e., the number of cycles that would produce a detectable quantity of cytokine product DNA that was directly proportional to the quantity of input mRNA) was determined experimentally. To verify that equal amounts of undegraded RNA were added to each PCR reaction within an experiment, the housekeeping gene, hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used as an endogenous internal standard and amplified with specific primers at the number of cycles at which a linear relationship between input RNA and final HPRT product was detected. Although HPRT values did not usually vary more than two- and threefold, values for specific cytokines are normalized to the HPRT values.

Southern blotting. From the final PCR reaction, 10 µl of solution was transferred to a new 0.5 ml tube containing 2 µl of loading buffer. The solution was denatured at 65°C for 5 min., placed on ice for 5 min., and loaded onto a 1% agarose-TBE gel. Following electrophoresis at 100V for approx. 45 min., the gel was soaked in a denaturing solution (1.5 M NaCl, 0.5 M NaOH at pH of 13) for 25 min. and the solution was replaced with a fresh batch. The gel was rinsed twice with distilled water and soaked in 20X SSPE for 30 min. A 0.2 µM Nytran membrane (Schleicher and Schuell, Inc., Keene, NH) was prepared by placing soaking it in distilled water with two pieces of GB002 thin blotting paper (Schleicher and Schuell, Inc.) 10X SSPE and one piece of GB004 thick blotting paper (Schleicher and Schuell, Inc.) 5X SSPE for 30 min. The gel was removed and placed upside down on the bench top covered with plastic wrap. The Nytran was put on top of the gel, the agarose was trimmed to fit and air bubbles were removed. The GB002 paper was placed above the Nytran followed by the GB004 paper. Above this, dry GB004 paper was added (about 3 inches) followed by a flat tray and several weights. The transfer was allowed to occur overnight.

Prehybridization. Following transfer, the DNA was allowed to crosslink in a UV Stratalinker 1800 (Stratagene, Menasha, WI). The prehybridization solution was prepared as follows: 30 ml of 20X SSPE, 10 ml of Denhardt's solution, 10 ml of 10% SDS and 50 ml of distilled water. To this solution, denatured salmon sperm DNA was

added to a final concentration of 50 μg/ml and this solution was heated to 42°C. The blot was arranged over a piece of nylon mesh and rolled to fit into the hybridization bottle. The blot was placed in the hybridization oven at 42°C for 5 hrs.

Probe labeling. The probe was end-labeled with [γ-^{32}P]-ATP. The probe buffer was prepared as follows: 5.0 μl of 1M Tris (pH 7.6); 0.5 μl of 2M $MgCl_2$; 1.0 μl of 0.5M dithiothreitol; and 3.5 μl of distilled water. The probe reaction mix was prepared as follows: 2.5 μl of probe buffer; 2.0 μl of oligonucleotide probe (0.2 μg/μl); 1.0 μl of T4 polynucleotide kinase; 9.5 μl of distilled water; and 10 μl of [γ-^{32}P]-ATP (10 mCi/ml). The probe reaction mix was incubated at 37°C for 40 minutes. The G-25 Sephadex spin column (5-prime-3-prime, Boulder, CO) was prepared by inverting several times, removing the cap and stopper, draining the column and centrifuging for 2 min. with a refrigerated Sorvall RC-3B. The probe reaction was added to the column and centrifuged again for 4 min. 1μl of the probe reaction was added to 5 ml of scintillation fluid and the ^{32}P incorporation was measured with a Beckman liquid Scintillation Counter (Beckman, Inc., Irvine, CA).

Hybridization. The hybridization mix was prepared as follows: 30ml of 20X SSPE, 10 ml of 10% SDS, 60 ml of distilled water. The solution was heated to 49°C and 15 x 10^6 cpm of probe was added to 10 ml of hybridization solution and placed in the

hybridization bottle. The hybridization bottle was placed in the oven on the rotating cycle and incubated overnight.

Blot washing. The blot was washed in a low-stringency washing solution at 49°C for 15 min. which consists of: 180 ml of 20X SSPE; 6 ml of 10% SDS; and 414 ml of distilled water. The washing was repeated in a high-stringency washing solution at 49°C for 45 sec. which is prepared as follows: 30 ml of 20X SSPE; 6 ml of 10% SDS; and 564 ml of distilled water.

PhosphoImager Analysis. Amplified PCR product was detected by Southern blot analysis, and the resultant signal was quantitated with a PhosphoImager (Molecular Dynamics, Sunnyvale, CA), which uses a phosphor screen to detect radioactive signals on the Southern blot. The quantity of gene expression for each cytokine was calculated by by normalizing each gene expression value to its corresponding HPRT value.

Statistical analysis of cytokine gene expression measurements. To analyze the cytokine gene expression data by RT-PCR, a log transformation was performed of the ratio of measurements for cytokine to the housekeeping gene HPRT mRNA. For each cytokine and treatment group, the mean of these values was determined, and the mean of the log-transformed ratio for the untreated values subtracted from these means. To compare treatment groups, a one-way analysis of variance was calculated on the log-transformed ratios. If the one-way analysis of variance was significant, then a Newman -

Keuls test was conducted to determine where the statistically significant differences among treatment groups existed. Data are calculated as the mean ± SEM , and statistical significance is assumed for p less than or equal to 0.05. For GC quantitation, ELISPOT, eosinophilia and mastocytosis studies, arithmetic means and SEM were calculated for five animals per group (in a few cases three animals per group were used with knockout mice). For serum Ig measurements, geometric means and SEM were calculated. All of the experiments shown have been repeated at least twice.

III. Results

A. Studies on the effects of blocking B7-1 and B7-2 interactions during a type 2 *in vivo* immune response

1. Administration of both anti-B7-1 and anti-B7-2 Abs inhibits *H. polygyrus*-induced elevations in serum IgG1, IgE, intestinal mastocytosis, and blood eosinophilia

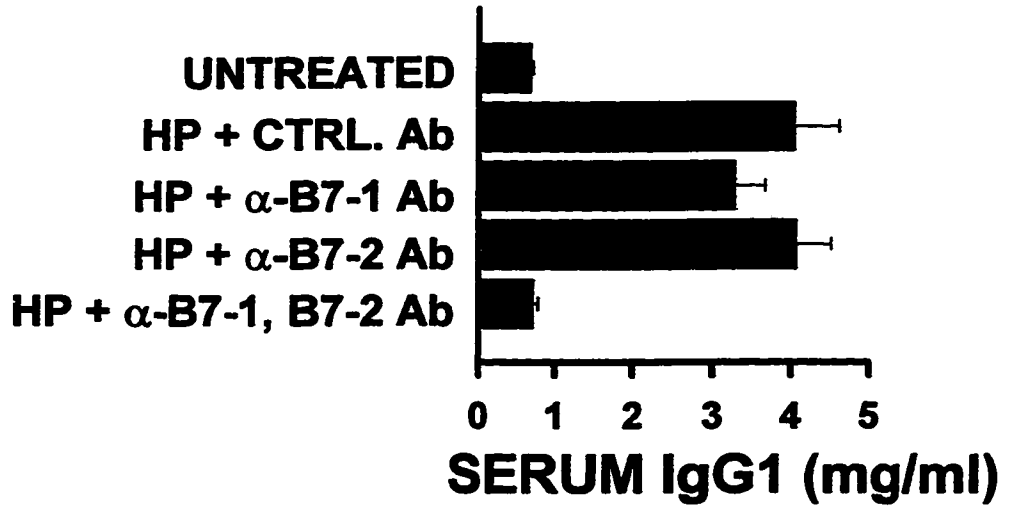
Previous studies in our laboratory have demonstrated that CTLA-4 ligand interactions are required for increased serum Ig levels during a type 2 primary *in vivo* immune response (Lu et al., 1994). To determine whether B7-1 and/or B7-2 ligand interactions are required for the development of allergy-associated effector cells during a type 2 immune response, BALB/c mice (five per treatment group) were orally inoculated with 200 third-stage *H. polygyrus* larvae and, on days 0 and 4, were injected i.v. with 100 μg of either anti-B7-1 or anti-B7-2 mABs, the combination of anti-B7-1 and anti-B7-2 mAbs, or control Igs. On day 14 after *H. polygyrus* inoculation, mice were killed, and serum IgG1, IgG2a, and IgE levels, blood eosinophils, and MMC were evaluated. Previous studies have shown that elevations in serum IgG1 and IgE levels, blood eosinophils, and MMC are readily detectable on day 14 after *H. polygyrus* inoculation (Lu et al., 1994; Svetic et al., 1993). *H. polygyrus*-inoculated mice administered control Igs or either anti-B7-1 or anti-B7-2 mAbs exhibit marked increases in serum IgG1 and IgE

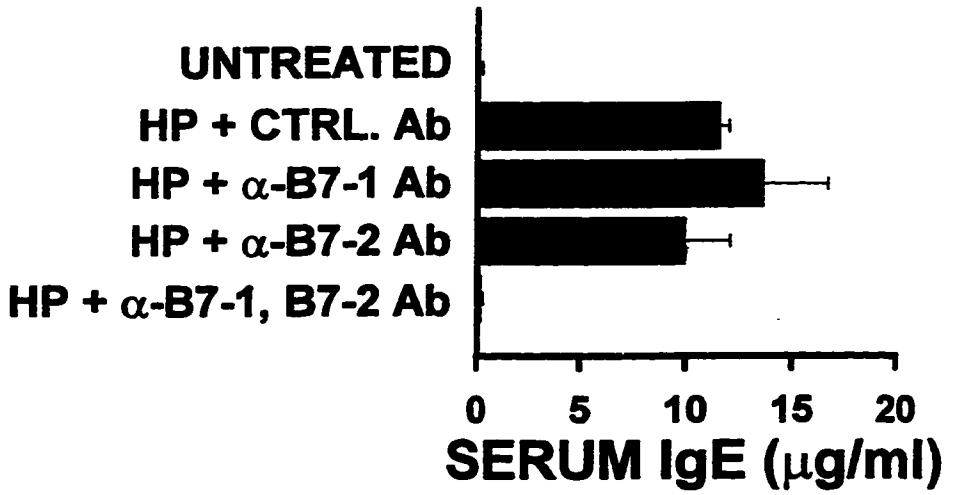
levels, MMC, and blood eosinophils (Figs. 4 and 5). In contrast, combined anti-B7-1 and anti-B7-2 Ab treatment in *H. polygyrus*-inoculated mice reduced serum IgG1 and IgE levels, blood eosinophils, and MMC to uninfected control levels. Elevations in serum IgG2a levels were not detected in any of the treatment groups, consistent with an inhibition of the response rather than with immune deviation. In this study, immune deviation would refer to a redirection of the immune response from a type 2 to a type 1 immune response and this did not occur in any of the treatment groups. These experiments were repeated two times with similar results.

2. The combination of anti-B7-1 and anti-B7-2 Abs is required to block GC formation and *in situ* expansion of CD4 $^{+}$ T cell populations

Previous studies have suggested that B7-2 is required for GC formation during a T-dependent immune response to a soluble Ag, suggesting that B7-2 may be the primary CD28 ligand required for the development of this lymphoid architecture (Han et al., 1995). To examine whether B7-1 and/or B7-2 are required for GC formation during a type 2 immune response to a pathogen, tissues were collected on day 8 from *H. polygyrus*-inoculated mice administered anti-B7-1 and/or anti-B7-2 Abs. In *H. polygyrus*-inoculated mice, well-formed, PNA-positive GCs were detected predominately in the follicular regions of the cortex. Markedly expanded $CD4^{+}$ T cell populations, detected with a mAb (L3T4), were observed in the T cell-rich region of the MLN cortex (Fig. 6). Increased GC size was similar in *H. polygyrus*-inoculated mice administered

Figure 4. Blocking both B7-1 and B7-2 inhibits elevations in serum IgG1 and IgE on day 14 after *H. polygyrus* inoculation. 100 μg of anti-B7-1 and/or anti-B7-2 mAbs or control (CTRL) Abs (100 μg of hamster Ig and 100 μg of control isotype-matched rat IgG2a) were administered on days 0 and 4 after oral inoculation with 200 third-stage *H. polygyrus* larvae. Mice were bled on day 14 after inoculation, and serum IgG1, IgE, and IgG2a levels were determined by ELISA. The mean and SE derived from serum samples of five individual BALB/c mice are shown for each treatment group.


UNTREATED
HP + CTRL. Ab
HP + α-B7-1 Ab
HP + α-B7-2 Ab
HP + α-B7-1, B7-2 Ab
0 1 2 3 4 5
SERUM IgG1 (mg/ml)


UNTREATED
HP + CTRL. Ab
HP + α-B7-1 Ab
HP + α-B7-2 Ab
HP + α-B7-1, B7-2 Ab
0 5 10 15 20
SERUM IgE (μg/ml)

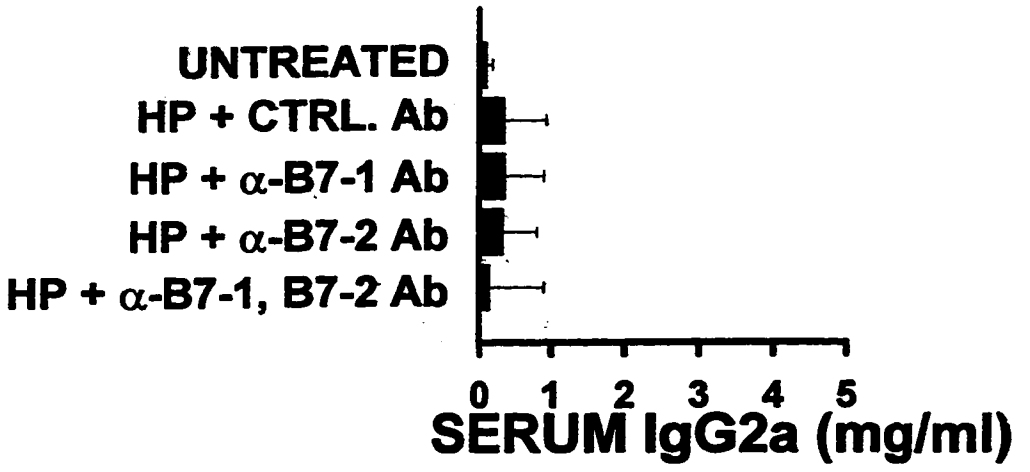
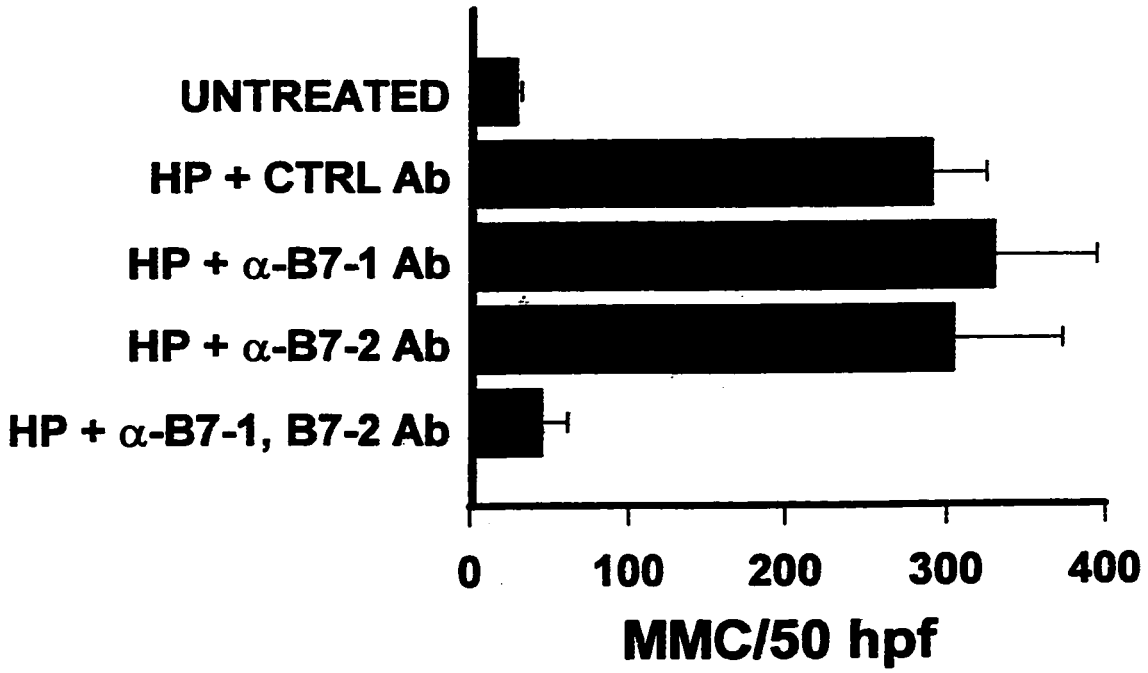

UNTREATED
HP + CTRL. Ab
HP + α-B7-1 Ab
HP + α-B7-2 Ab
HP + α-B7-1, B7-2 Ab
0 1 2 3 4 5
SERUM IgG2a (mg/ml)

Figure 5. The combination of anti-B7-1 and anti-B7-2 mAb treatment is required to inhibit elevations in intestinal MMC and blood eosinophils on day 14 after *H. polygyrus* (HP) inoculation. One hundred micrograms of anti-B7-1 and/or anti-B7-2 mAbs or control (CTRL) Abs (100 μg of hamster Ig and 100μg of control isotype-matched rat IgG2a) were injected on days 0 and 4 after oral inoculation with *H. polygyrus*. Mice were bled on day 14 after inoculation, intestinal tissue was removed and prepared for histological analysis, and blood eosinophil counts and the numbers of intestinal mucosal mast cells per 50 high powered microscope fields (hpf) were determined in samples of individual mice as described in *Materials and Methods*. The mean and SE are shown for intestinal samples of five individual BALB/c mice per treatment group. The experiments shown were repeated two times with similar results.


UNTREATED
HP + CTRL Ab
HP + α-B7-1 Ab
HP + α-B7-2 Ab
HP + α-B7-1, B7-2 Ab
0
100
200
300
400
MMC/50 hpf

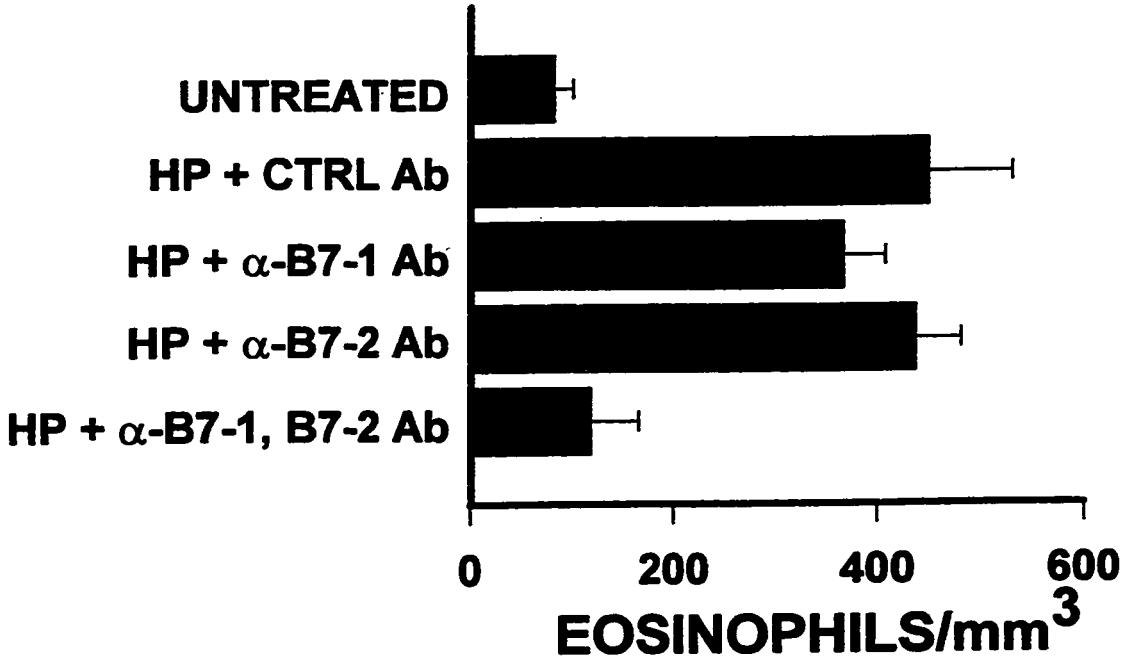
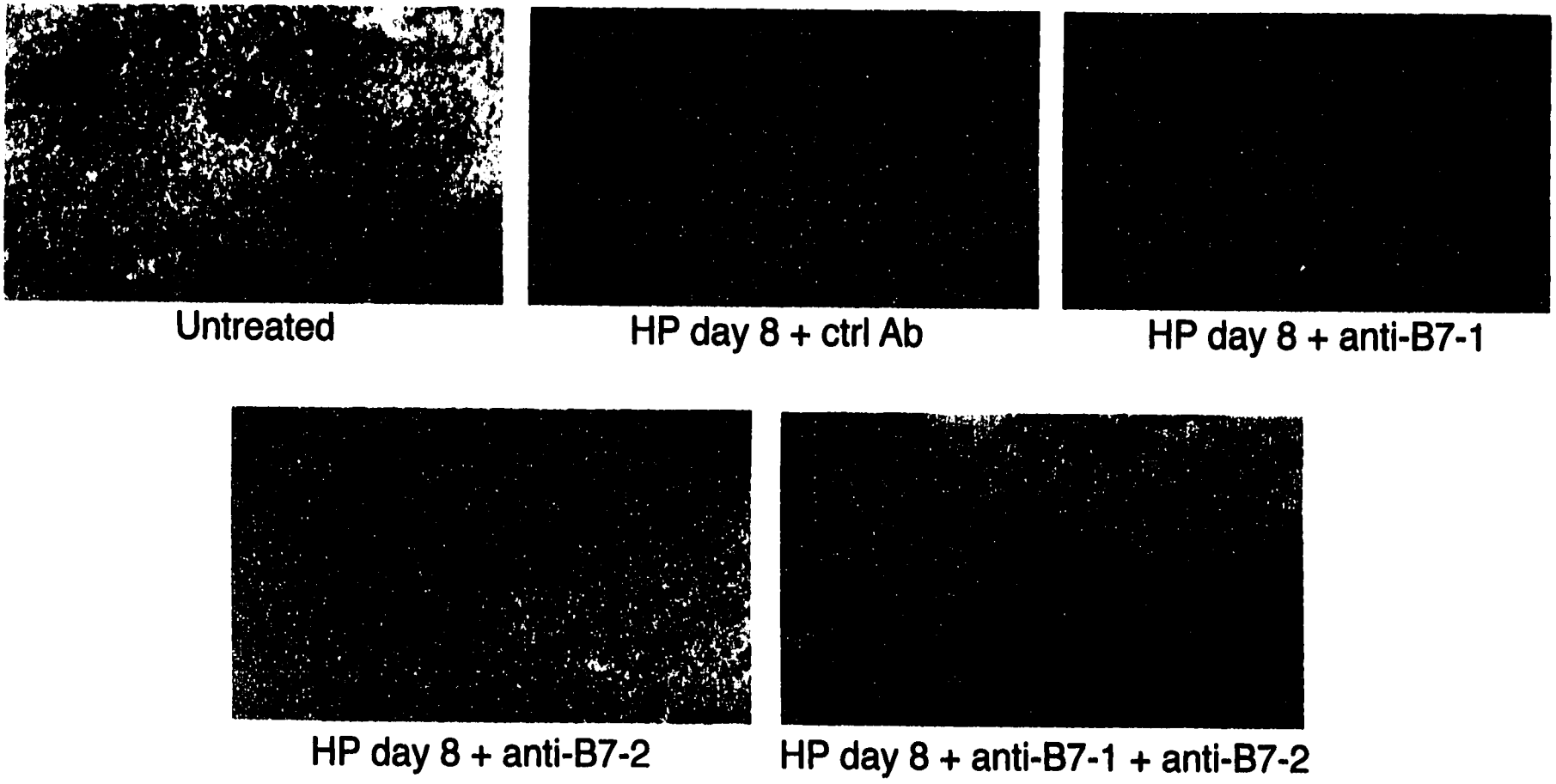

UNTREATED
HP + CTRL Ab
HP + α-B7-1 Ab
HP + α-B7-2 Ab
HP + α-B7-1, B7-2 Ab
0
200
400
600
EOSINOPHILS/mm3

Figure 6. $CD4^+$ T cell expansion and increased germinal center formation are blocked in *H. polygyrus*-inoculated mice. One hundred micrograms of anti-B7-1 and/or anti-B7-2 mAbs or a combination of control Abs were injected on days 0 and 4 after oral inoculation with *H. polygyrus*. Mice were either untreated (A) or inoculated with *H. polygyrus* and treated with control Abs (B), anti-B7-1 (C), anti-B7-2 (D), or both anti-B7-1 and anti-B7-2 mAbs (E). MLNs were collected on day 8 from BALB/c mice (five mice per treatment group), frozen in liquid nitrogen, and subsequently stained at 8 μm. Tissue sections were stained for $CD4^+$ T cells with GK1.5 (blue stain) and for GC cells with the lectin, PNA (red stain). Untreated controls exhibited little GC formation (A). *H. polygyrus*-inoculated mice treated with control Abs (B) exhibited marked increases in GC formation, while inoculated mice given either anti-B7-1 (C) or anti-B7-2 (D) showed GC expansion that was comparable to *H. polygyrus*-inoculated mice given control Abs. Administration of both anti-B7-1 and anti-B7-2 mAbs (E) to inoculated mice abolished GC formation. The tissue sections were analyzed by light microscopy at a low magnification (approx. X125), and each panel is representative of the average field. This experiment was repeated two times with similar results.


Untreated
HP day 8 + ctrl Ab
HP day 8 + anti-B7-1
HP day 8 + anti-B7-2
HP day 8 + anti-B7-1 + anti-B7-2

control, anti-B7-1, or anti-B7-2 Abs. In marked contrast, blocking B7-1 and B7-2 ligand interactions with the combination of both anti-B7-1 and anti-B7-2 Abs inhibited both *H. polygyrus*-induced increases in GC formation and *in situ* $CD4^+$T cell expansion (Fig. 6). In summary, these results indicate that either B7-1 or B7-2 can provide the costimulatory signal required for increased GC formation and $CD4^+$ T cell expansion in the MLN following *H. polygyrus* inoculation. These experiments were repeated twice with similar results.

In another experiment, GC formation was quantitated in the MLN of *H. polygyrus*-inoculated mice administered 200 μg of anti-B7-1 and/or anti-B7-2 Abs on day 0 and 4 and killed on day 8 after inoculation. Quantitative volumetric analyses were performed with individual MLN tissues (five mice per treatment group), and the mean GC volume was determined for each tissue. As shown in Table II, *H. polygyrus*-inoculated mice administered both anti-B7-1 and anti-B7-2 mAbs showed an inhibition of GC formation to levels of untreated controls. Furthermore, as observed with doses of 100μg of anti-B7 mAbs, $CD4^+$ T cell expansion was reduced to untreated control levels in inoculated mice given anti-B7-1 and anti-B7-2 mAbs, but was not affected in mice given either anti-B7 Ab alone.

Table II. Effect of anti-B7-1 and anti-B7-2 mAbs on GC formation at day 8 after *H. polygyrus* inoculation

Groups	GC volume (%)[a]
Untreated	< 5%
HP + control Abs[b]	29.5 - 36.7%
HP + anti-B7-1 Ab[c]	31.6 - 36.6%
HP + anti-B7-2 Ab[c]	23.9 - 27.3%
HP + anti-B7-1 + anti-B7-2 Abs[c]	< 5%

[a] Mesenteric lymph node PNA^+ GCs were quantified volumetrically for each animal as described in the *Materials and Methods.* A value of less than 5% represents minimal GC formation. Values are expressed as a percentage range for five individual mice per group.

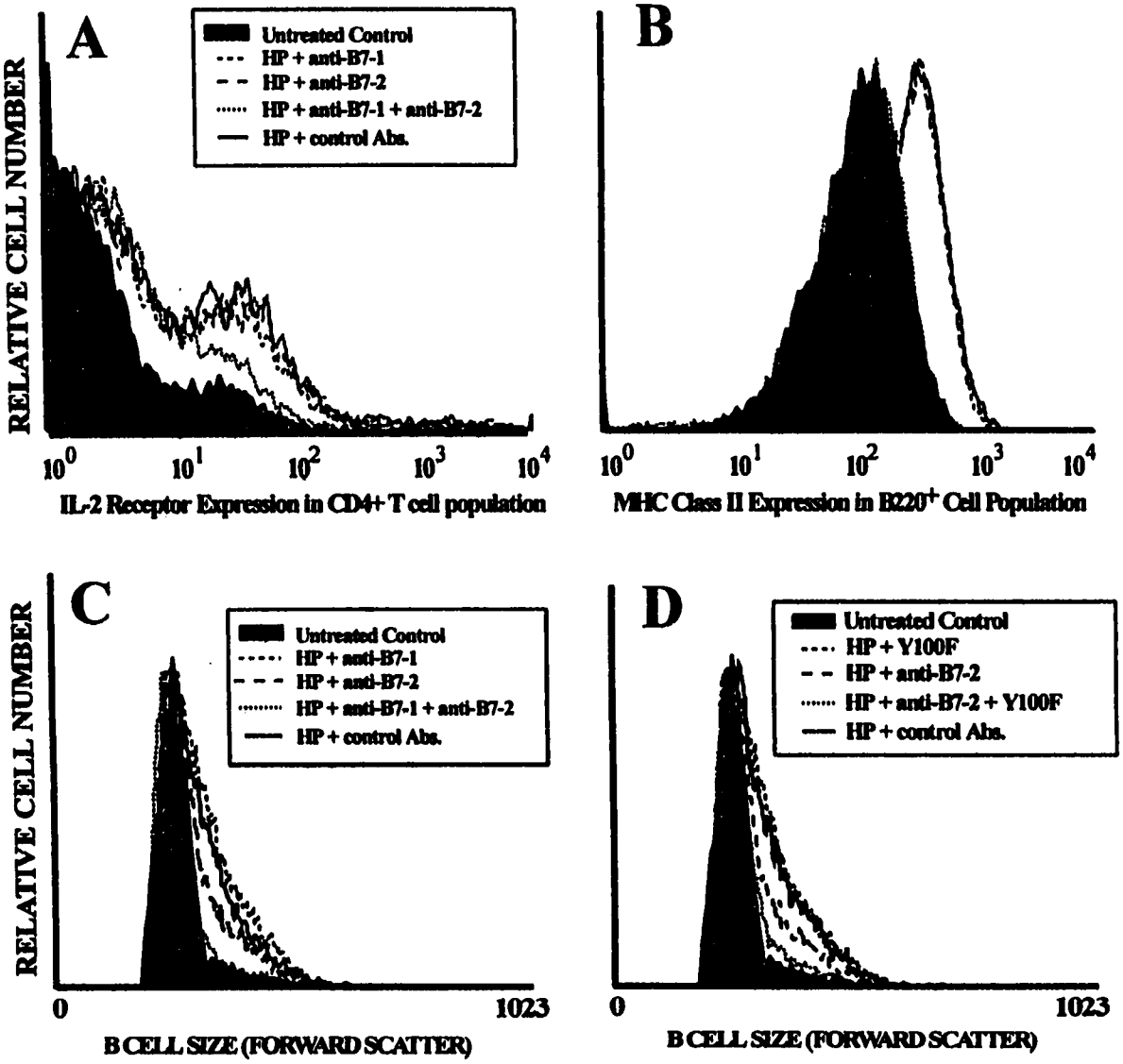
[b] Control mice were administered 200 μg of GL117 Ab and normal hamster IgG on days 0 and 4 after HP inoculation.

[c] Mice were injected with 200 μg of anti-B7-1 and/or anti-B7-2 mAbs on days 0 and 4 after HP inoculation.

3. Administration of both anti-B7-1 and anti-B7-2 Abs blocks B cell activation, leading to increased cell size and MHC class II surface expression and inhibits increases in $CD4^+$ T cell IL-2R expression.

The inhibition of GC formation on day 8 and the suppression of serum IgE and IgG1 elevations on day 14 in *H. polygyrus*-inoculated mice injected with both anti-B7-1 and anti-B7-2 mAbs suggested that blocking B7 interactions may affect B cell activation and blastogenesis. To assess B cell activation in *H. polygyrus*-inoculated mice treated with 100 μg of anti-B7-1 and/or anti-B7-2 mAbs, MLN cell suspensions from each animal were dual stained with anti-MHC class II and B cell-specific anti-B220 (6B2) mAbs. MHC class II expression and forward light scatter (a correlate of cell size) of $B220^+$ cells were markedly increased in *H. polygyrus*-inoculated mice given control Abs, whereas mice injected with both anti-B7-1 and anti-B7-2 mAbs exhibited decreased MHC class II and forward light scatter profiles. Blocking either B7-1 or B7-2 alone had little effect (although anti-B7-2 mAb alone showed some partial decrease in B cell size), but the combined inhibition of B7-1 and B7-2 interactions caused marked decreases in MHC class II expression and B cell size (Fig. 7B). On day 8 after *H. polygyrus* inoculation, MLN cells were also dual-stained with FITC-anti-CD4 and CY5 anti-IL-2R mAb. The combination of anti-B7-1 and anti-B7-2 mAbs, but neither antibody alone, partially blocked increases in $CD4^+$ T cell IL-2R expression (Fig. 7A). Comparable results were obtained when *H. polygyrus*-inoculated mice (five mice per treatment group) were administered 200 μg of anti-B7-1 and/or anti-B7-2 mAbs.

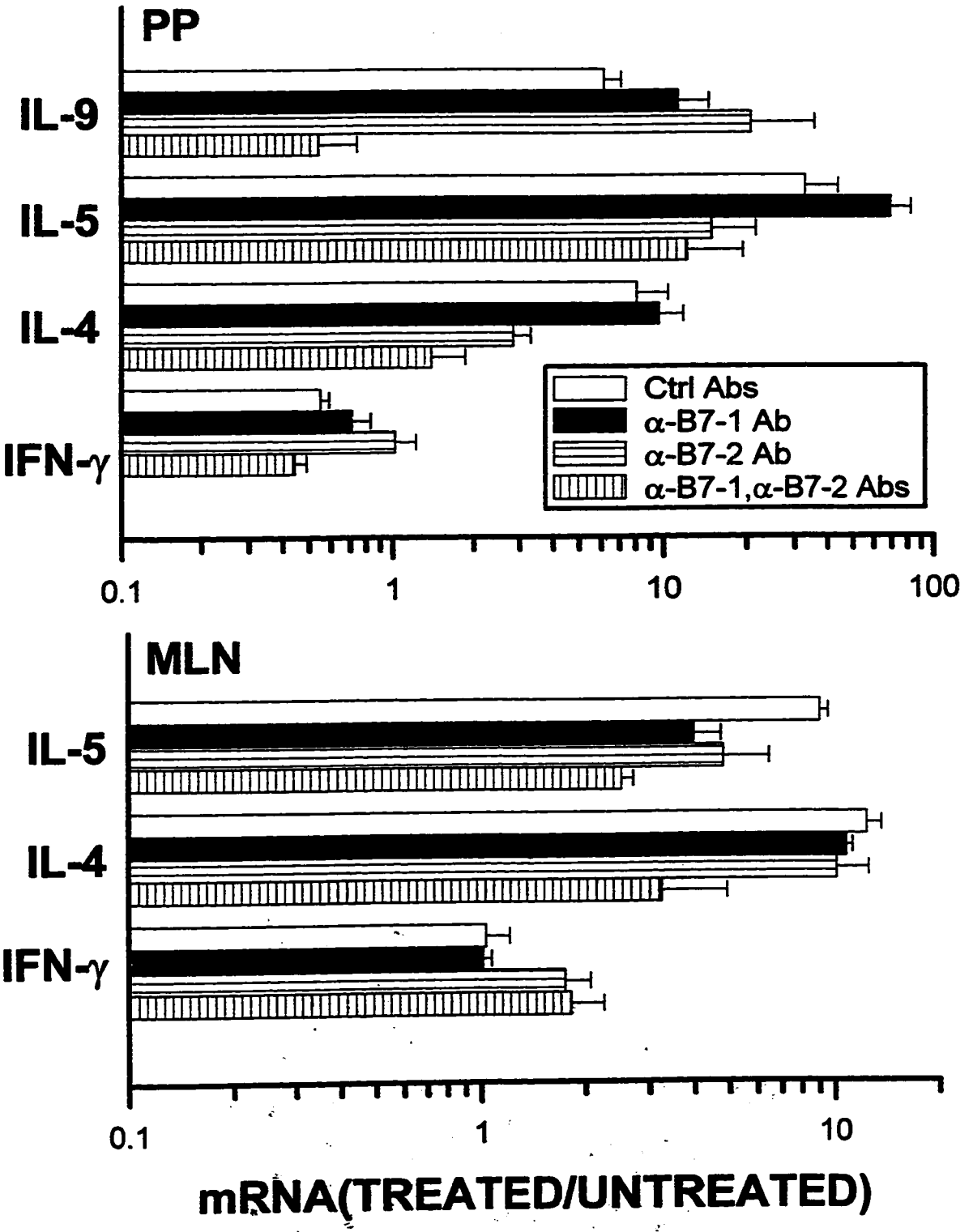
Figure 7. Increases in T cell IL-2R expression and B cell size are blocked only by inhibiting both B7-1 and B7-2 interactions during the immune response to *H. polygyrus* (HP). 100 μg of anti-B7-1 and/or anti-B7-2 mAbs or control Abs (100 μg of hamster Ig and 100 μg of control isotype-matched rat IgG2a) were administered on days 0 and 4 after oral inoculation with 200 third-stage *H. polygyrus* larvae. MLNs were collected on day 8 after inoculation, and cell suspensions from five individual BALB/c mice per treatment group were pooled and dual stained with FITC-anti-CD4 and CY5-anti-IL-2R mAb or FITC-anti-MHC class II and biotinylated anti-B220 (6B2) followed by phycoerythrin-avidin. Single histogram analyses of $CD4^+$ T cell IL-2Rα expression (A), B cell MHC class II expression (B), and B cell size (forward scatter analysis) (C) are shown. In panel (D), the protocol was the same as that for panels A-C, except that Y100F was substituted for anti-B7-1 mAb. A single histogram forward scatter analysis of $B220^+$ B cells is shown.


A
Untreated Control
HP + anti-B7-1
HP + anti-B7-2
HP + anti-B7-1 + anti-B7-2
HP + control Abs.
RELATIVE CELL NUMBER
10^0 10^1 10^2 10^3 10^4
IL-2 Receptor Expression in CD4+ T cell population
B
10^0 10^1 10^2 10^3 10^4
MHC Class II Expression in B220+ Cell Population
C
Untreated Control
HP + anti-B7-1
HP + anti-B7-2
HP + anti-B7-1 + anti-B7-2
HP + control Abs.
RELATIVE CELL NUMBER
0
1023
B CELL SIZE (FORWARD SCATTER)
D
Untreated Control
HP + Y100F
HP + anti-B7-2
HP + anti-B7-2 + Y100F
HP + control Abs.
0
1023
B CELL SIZE (FORWARD SCATTER)

4. Administration of both anti-B7-1 and anti-B7-2 Abs inhibits elevations in type 2 cytokines in both the Peyer's patch (PP) and MLN of *H. polygyrus*-inoculated mice.

The requirement for both B7-1 and B7-2 interactions for inhibition of B cell activation and differentiation during the immune response to *H. polygyrus* suggested that T cell activation leading to cytokine production may also be blocked only by the combined administration of both anti-B7-1 and anti-B7-2 mAbs. Previous studies have shown that by day 8 after *H. polygyrus* inoculation, elevations in IL-5 and IL-9 mRNA are partly T cell dependent, while the sole source of IL-4 elevations is TCR $\alpha\beta^+$, $CD4^+$ T cells (Svetic et al., 1993). To identify the requirement of B7-1 and/or B7-2 ligands for increased type 2 cytokine gene expression, *H. polygyrus*-inoculated mice were injected with anti-B7-1 and/or anti-B7-2 mAbs on days 0 and 4. On day 8 after inoculation, the PP and MLN were collected from the mice (five per group) and analyzed for cytokine gene expression by quantitative RT-PCR. All treatment groups were expressed as the treated:untreated ratio, resulting in the untreated being equal to a value of 1. In the MLN, statistically significant (p value < 0.05) decreases in IL-4 and IL-5 were observed in *H. polygyrus*-inoculated mice administered both anti-B7-1 and anti-B7-2 mAbs compared with levels in inoculated mice administered control Abs (Fig. 8). In contrast, no significant differences were detected when *H. polygyrus*-inoculated mice were given either anti-B7-1 or anti-B7-2 mAbs alone. IFN-γ and IL-2 were not significantly changed in any of the treatment groups, as they varied less than twofold from untreated control values. IL-9 elevations are not usually detectable in the MLN (data not shown). In the

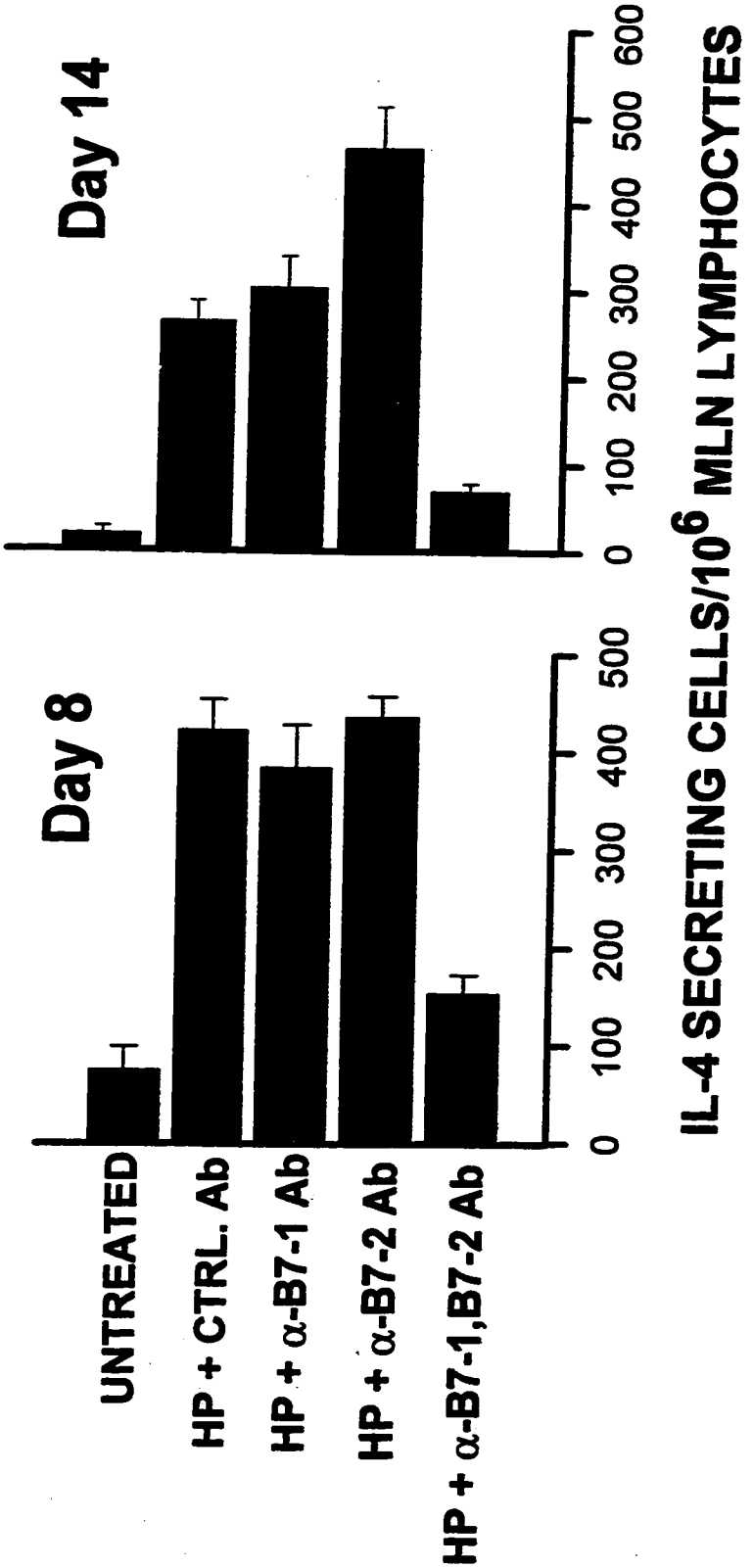
Figure 8. The combination of anti-B7-1 and anti-B7-2 mAbs inhibits elevations in Th2 cytokine gene expression in the PP and MLN on day 8 after *H. polygyrus* inoculation. One hundred micrograms of anti-B7-1 and/or anti-B7-2 or control (CTRL) Abs were administered on days 0 and 4 to *H. polygyrus*-inoculated mice. PP and MLN tissues were collected on day 8 after inoculation, and cytokine gene expression was determined by quantitative RT-PCR. The mean and SE are derived from RNA preparations from the PP and MLN of five individual mice normalized to the internal standard, HPRT, which did not show more than two- to threefold changes throughout the experiment. The means are expressed relative to the mean of the uninfected control, which was arbitrarily given a value of 1. Similar results were obtained in two independent experiments.


PP
IL-9
IL-5
IL-4
IFN-γ
Ctrl Abs
α-B7-1 Ab
α-B7-2 Ab
α-B7-1,α-B7-2 Abs
0.1
1
10
100
MLN
IL-5
IL-4
IFN-γ
0.1
1
10
mRNA(TREATED/UNTREATED)

PP, significant decreases (p value < 0.05) were detected in IL-4 and IL-9, and partial, but not significant, decreases were observed in IL-5 gene expression in *H. polygyrus*-inoculated mice administered both anti-B7-1 and anti-B7-2 mAbs compared with inoculated mice administered control Abs. In addition, IL-4 was also significantly reduced in *H. polygyrus*-inoculated mice administered anti-B7-2 mAb alone. IFN-γ and IL-2 gene expression remained at or below untreated control levels in all treatment groups. The partial inhibitory effect of anti-B7 mAb treatment on IL-5 elevations may be due to contributions from non-T cells. Previous studies have shown that *H. polygyurs*-inoculated mice administered anti-CD4 antibodies show only partial decreases in IL-5 gene expression (although IL-4 is completely blocked) at day 8 after inoculation (Svetic et al. 1993, Lu et al., 1994).

To identify the requirement for B7-1 and/or B7-2 ligands for the development of IL-4 secretion, *H. polygyrus*-inoculated mice were treated with 100 μg of anti-B7-1 and/or anti-B7-2 mAbs on both days 0 and 4. On days 8 and 14 after *H. polygyrus* inoculation, MLN were collected (five mice per group) and analyzed for protein secretion by ELISPOT assays. Treatment with both anti-B7-1 and anti-B7-2 mAbs reduced the number of IL-4 secreting cells to slightly above untreated levels on days 8 and 14 (Fig. 9). Treatment with either anti-B7-1 or anti-B7-2 Ab alone did not reduce elevations in IL-4 secretion compared with that in *H. polygyrus*-inoculated mice administered control Abs. Although increases were detected in IL-5 secretion following *H. polygyrus* inoculation, statistically significant decreases in IL-5 were not detected in any of the

Figure 9: The combination of anti-B7-1 and anti-B7-2 mAbs inhibits elevations in IL-4 secretion on days 8 and 14 in the MLN of *H. polygyrus* (HP)-inoculated mice. Mice were orally inoculated with 200 third-stage *H. polygyrus* larvae and injected with 100μg of anti-B7-1 and/or anti-B7-2 mAbs or control (CTRL) Abs on days 0 and 4. MLN tissues were collected on days 8 and 14 after inoculation, and the number of IL-4 secreting cells per 10^6 MLN cells was determined in an ELISPOT assay without restimulation. Cell suspensions were assayed from five individual BALB/c mice per treatment group, and the mean and SE are shown. Similar results were obtained in several independent experiments.


Day 14
Day 8
0
100
200
300
400
500
600
UNTREATED
HP + CTRL. Ab
HP + α-B7-1 Ab
HP + α-B7-2 Ab
HP + α-B7-1,B7-2 Ab
IL-4 SECRETING CELLS/10^6 MLN LYMPHOCYTES

treatment groups, consistent with previous observations that IL-5 is derived from non-T cells as well as T cells and with the previous observation that CTLA-4Ig only partially inhibits IL-5 gene expression and secretion (Lu et al., 1994). Elevations in MLN IFN-γ secretion beyond that in untreated controls were not detected in any of the treatment groups, consistent with the observation that serum IgG2a levels were also not elevated. Similar results were obtained when *H. polygyrus*-inoculated mice were administered 200 μg of anti-B7-1 and/or anti-B7-2 mAbs. These experiments were repeated two times with similar results.

5. The combination of both anti-B7-1 and anti-B7-2 or Y100F and anti-B7-2 blocks elevations in IL-4 secretion and B cell activation on day 8 after *H. polygyrus* inoculation

The recent identification of distinct epitopes on CTLA-4 that bind either B7-1 and/or B7-2 (Linsley et al., 1994) allowed the construction of a chimeric mutated CTLA-4Ig fusion protein that specifically blocked B7-1, but not B7-2, interactions with either CTLA-4 or CD28 (Peach, personal communication). This mutant CTLA-4 (Y100F) has a single amino acid substitution of tyrosine for phenylalanine at position 100. Y100F was also considered a useful control for possible non-specific effects of the hamster anti-B7-1 mAb. To test the specificity of this mutant CTLA-4 fusion protein for B7-1, binding of Y100F to Chinese hamster ovary cells, transfected with either B7-1 or B7-2 was assessed by R. Peach. Y100F was unable to bind to the human or murine B7-2 transfectants, but did effectively bind B7-1 transfectants (R. Peach, unpublished observations). To

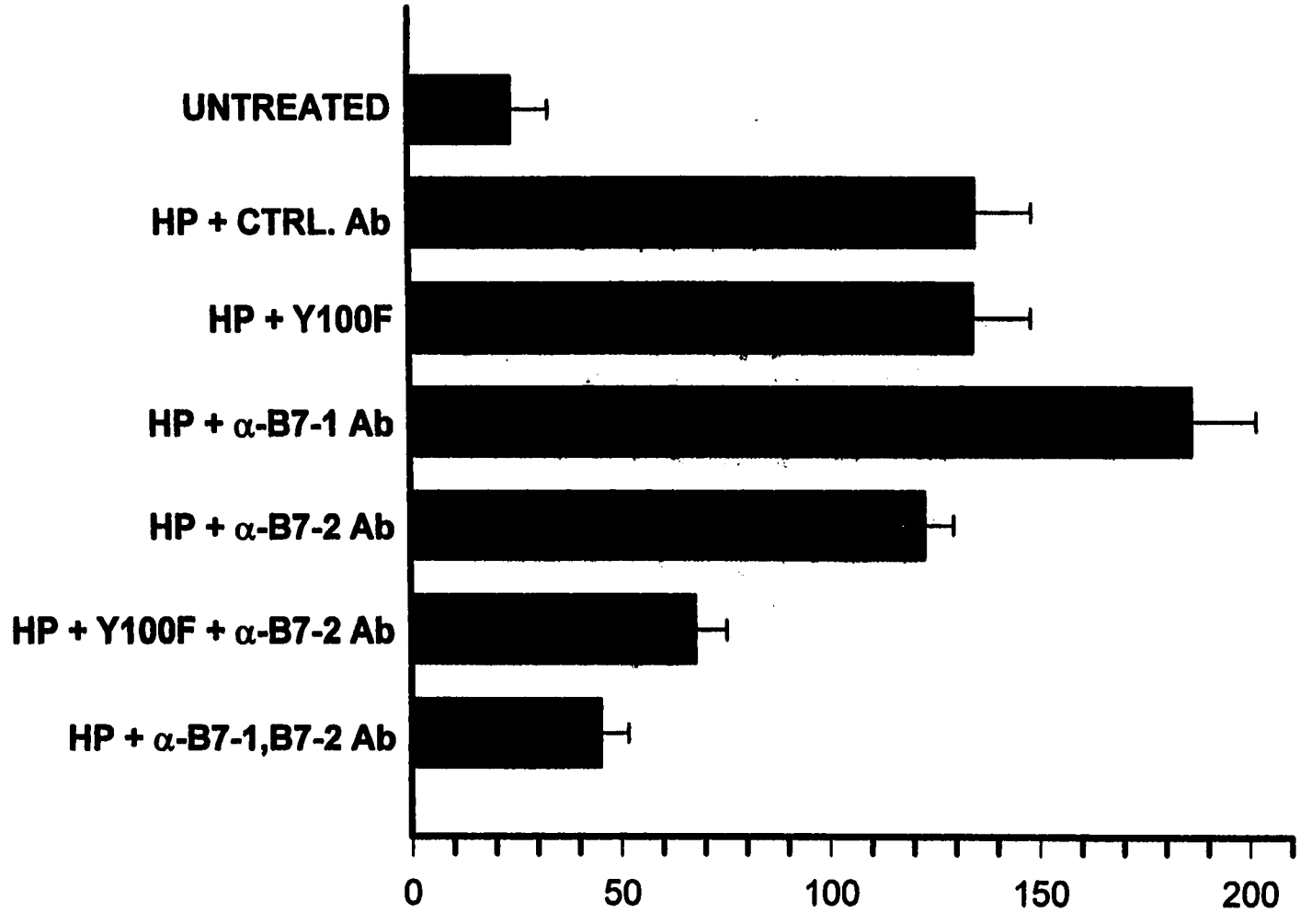
determine whether this novel B7-1-specific CTLA-4Ig fusion protein functioned similarly to anti-B7-1 mAb in vivo, *H. polygyrus*-inoculated mice (five per group) were administered anti-B7-1, anti-B7-2, Y100F, anti-B7-2 plus anti-B7-1, or anti-B7-2 plus Y100F. MLNs were collected on day 8 after *H. polygyrus* inoculation and analyzed by the ELISPOT assay for IL-4 secretion (Figure 10). Only the combination of either anti-B7-1 or Y100F plus anti-B7-2 blocked elevations in IL-4 secretion. Similarly, increases in B cell size (as measured by forward scatter analysis) were blocked to untreated control levels when the combination of anti-B7-1 and anti-B7-2 or Y100F and anti-B7-2 mAbs was administered to *H. polygyrus*-inoculated mice (Fig. 7d). Taken together, these studies suggest that Y100F and hamster anti-B7-1 mAb have similar effects in blocking B7-1 interactions during the primary mucosal type 2 immune response to this pathogen.

B. Increased B7-2 dependence with the progression of a chronic type 2 *in vivo* immune response

1. GC formation is pronounced in B7-2WT as well as B7-2KO mice following *H. polygyrus* inoculation and is B7-1-dependent.

Previous studies with blocking anti-B7-2 antibodies have indicated that B7-2 ligand interactions are required for the development of GCs in a T-dependent humoral immune response to a soluble protein antigen (Han et al., 1995), and other studies have

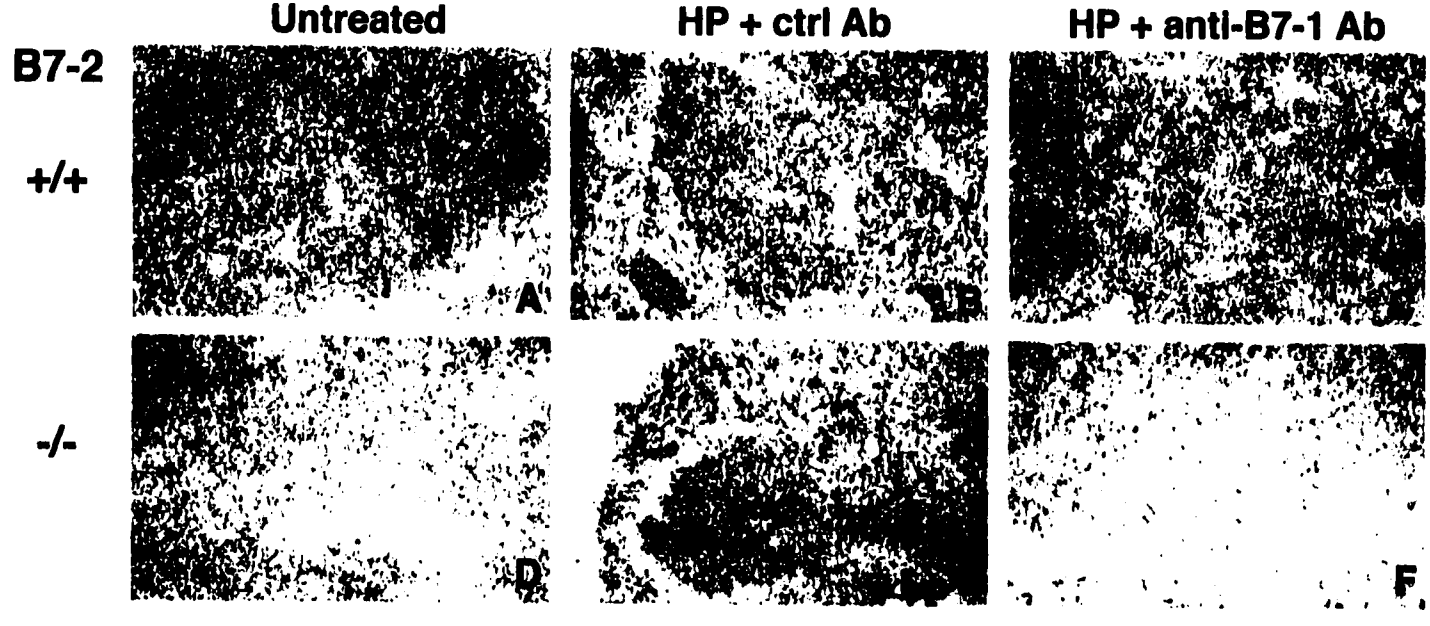
Figure 10. The combination of anti-B7-2 and either anti-B7-1 or Y100F blocks elevations in IL-4 secretion on day 8 after *H. polygyrus* (HP) inoculation. One hundred micrograms of anti-B7-1 and/or anti-B7-2 mAbs, Y100F and/or anti-B7-2 mAb, or control (CTRL) Abs were administered on days 0 and 4 to *H. polygyrus*-inoculated mice. MLN tissues were removed on day 8 after inoculation, and the number of IL-4 secreting cells per 10^6 MLN cells was determined by an ELISPOT assay without re-stimulation. The mean and SE are shown for five individual mice per treatment group. This experiment was repeated two times with similar results.



CYTOKINE SECRETING CELLS/10^6 MLN LYMPHOCYTES

suggested that B7-2 ligand interactions favor a type 2 immune response (Kuchroo et al., 1995; Ranger et al., 1996). We have recently demonstrated that administration of both anti-B7-1 and anti-B7-2 antibodies are required to inhibit GC formation during the mucosal immune response that follows oral inoculation with *H. polygyrus* (Greenwald et al., 1997). Since anti-B7-2 antibodies may incompletely inhibit B7-2 ligand interactions in peripheral mucosal lymphoid tissues, a particular problem at later stages of the response when anti-Ig antibody may increase clearance and inhibit binding of the anti-B7-2 antibody, we examined whether the GC reaction during the mucosal immune response to *H. polygyrus* occurs in B7-2-deficient mice. BALB/c Wild-type (WT) (five mice per treatment group) and BALB/c B7-2 knockout (KO) mice (four mice per treatment group) were orally inoculated with 200 third-stage *H. polygyrus* larvae. Eight and fourteen days after inoculation, control and infected mice were sacrificed and MLN tissues removed, sectioned and dual-stained with PNA and anti-CD4 mAb to detect increases in GC size and *in situ* $CD4^+$ T cell expansion, respectively. In *H. polygyrus*-inoculated B7-2KO mice, elevations in GC size and *in situ* $CD4^+$ T cell expansion were comparable to inoculated B7-2WT mice, suggesting that B7-2 ligand interactions are not required for lymphocyte differentiation leading to the formation of GCs in *H. polygyrus*-inoculated mice (Fig. 11B, E). To assess overall GC volume in the MLN, quantitative volumetric analyses was performed with individual MLN tissues, and the mean GC volume was determined for each tissue. As shown in Table III, *H. polygyrus*-inoculated B7-2WT and B7-2KO mice exhibited statistically similar GC volumes (~30-35%).

Figure 11. B7-2KO and B7-2WT mice exhibit comparable and pronounced germinal center (GC) formation after *H. polygyrus* inoculation, which can be inhibited in infected B7-2KO mice given anti-B7-1 mAb. 100 μgs of anti-B7-1 or ctrl Abs were administered on days 0 and 4 after oral inoculation with 200 third stage *H. polygyrus* larvae. MLNs were collected on day 14 from mice (5 per treatment group), frozen in liquid nitrogen and subsequently sectioned at 8 μm. Tissues were stained for $CD4^+$ T cells with GK1.5 (blue stain) and for GC with the lectin, PNA (red stain). Untreated controls and *H. polygyrus*-inoculated B7-2KO mice administered anti-B7-1 mAb exhibited little GC formation while all other treatment groups exhibited marked increases in GC formation.


Untreated
HP + ctrl Ab
HP + anti-B7-1 Ab
B7-2
+/+
-/-
A
B
C
D
E
F

It was possible that signaling molecules besides B7 ligands provided the cellular interactions required for GC formation in B7-2KO mice. To examine whether B7-1 provided the costimulatory signal required for GC formation in B7-2 deficient mice, B7-2KO mice were administered anti-B7-1 mAbs at day 0 and 4 after *H. polygyrus* inoculation. In marked contrast to the elevations in GC size and *in situ* $CD4^+$ T cell staining observed in *H. polygyrus*-inoculated B7-2KO mice administered control antibodies, blocking B7-1 ligand interactions in B7-2KO mice ablated *H. polygyrus*-induced elevations in these parameters (Fig. 11F, Table III). In addition, GC formation was similar in B7-2WT mice administered either anti-B7-1 mAb or control antibodies after *H. polygyrus* inoculation. These results suggest that B7-1 alone can provide the costimulatory signal through CD28/CTLA-4 interactions required for MLN GC formation and $CD4^+$ T cell expansion during the type 2 immune response to *H. polygyrus*. The GC reaction was also assessed at day 14 after *H. polygyrus* inoculation. Comparable increases in GC formation were observed in *H. polygyrus*-inoculated B7-2WT and B7-2KO mice (Table III).

2. Type 2 cytokines are comparably elevated in B7-2KO and WT mice at day 8 but not day 14 after *H. polygyrus* inoculation.

Some studies have suggested that B7-2 may or not be required for the development of the *in vivo* Th2 response. We have previously shown that administration of both anti-B7-1 and anti-B7-2 blocking antibodies are required to inhibit the *H. polygyrus* immune

Table III. GC formation at day 8 and 14 after *H. polygyrus* inoculation

Groups[a]	GC volume (%)[b]	
	Day 8	Day 14
B7-2WT:		
Untreated	< 5%	< 5%
HP + control Ab	32.3-40.5%	25.3-34.5%
HP + anti-B7-1 Ab	29.5-36.3%	29.4-33.0%
B7-2KO:		
Untreated	< 5%	< 5%
HP + control Ab	25.0-34.6%	30.2-38.4%
HP + anti-B7-1 Ab	< 5%	< 5%

[a] *H. polygyrus*-inoculated BALB/c B7-2WT and B7-2KO mice (5 per treatment group) were administered 200 μg of anti-B7-1 Ab or normal hamster IgG on days 0 and 4 for the day 8 time point and days 0, 4 and 7 for day 14.

[b] Mesenteric lymph node PNA^+ GCs were quantified volumetrically for each animal as described in the *Materials and Methods.* A value of less than 5% represents minimal GC formation. Values are expressed as a range of percentage values.

response (Greenwald et al., 1997). However, the exogenously administered anti-B7-2 antibodies may inhibit B7-2 interactions incompletely, a particular problem at later stages of the response when antibody responses against the foreign immunoglobulins may directly block their interactions with B7-2. To directly test the requirement of B7-2 for the *H. polygyrus* Th2 response, B7-2KO mice were orally inoculated with 200 third-stage *H. polygyrus* larvae and at days 8 and 14 mice were killed and assayed for Th2 cell cytokine production. Previous studies have shown that by day 8 after *H. polygyrus* inoculation, elevations in IL-5 and IL-9 mRNA are partly T cell dependent, while the sole source of IL-4 elevations is TCR-$\alpha\beta^+$,$CD4^+$ T cells (Svetic et al., 1993). On day 8 after inoculation, the Peyer's patch (PP) and mesenteric lymph node (MLN) were collected from the mice (5 mice per treatment group) and analyzed for cytokine gene expression by quantitative RT-PCR. As shown in Fig. 12, T cell derived IL-4 gene expression at day 8 is inhibited in *H. polygyrus*-inoculated B7-2KO mice administered anti-B7-1 mAbs as compared to the elevated levels of IL-4 gene expression in inoculated B7-2KO mice. By day 14, however, levels of IL-4 gene expression are comparable in *H. polygyrus*-inoculated B7-2KO mice administered control Abs or anti-B7-1 Abs (Fig. 12, 13). All treatment groups were expressed as the treated:untreated ratio, resulting in the untreated control being equal to a value of 1.

Elevations in IL-4 protein secretion as measured by an ELISPOT assay were also examined at day 8 after *H. polygyrus* inoculation. As shown in Fig. 14, IL-4 secretion

Figure 12. Elevations in IL-3, IL-4, IL-5, and IL-13 gene expression occur in *H. polygyrus*-inoculated B7-2KO and B7-2WT mice. For the determination of cytokine gene expression, tissues were collected at 8 days after inoculation and cytokine gene expression levels were then determined by a quantitative RT-PCR. All data were individually normalized to the internal standard, HPRT, which did not show greater than 2-3-fold changes throughout the experiment. Although significant elevations in IL-3, IL-4, IL-5, and IL-13 gene expression occurred in *H. polygyrus*-inoculated B7-2WT and B7-2KO mice as compared to untreated controls, IL-4 gene expression in inoculated B7-2KO mice administered anti-B7-1 Abs appears to be partially inhibited by (three-fold), but is not statistically significant. The means are expressed relative to the mean of the uninfected control, which was arbitrarily given a value of 1. The mean and standard error derived from individually assayed MLNs of five BALB/c mice are shown for each treatment group.

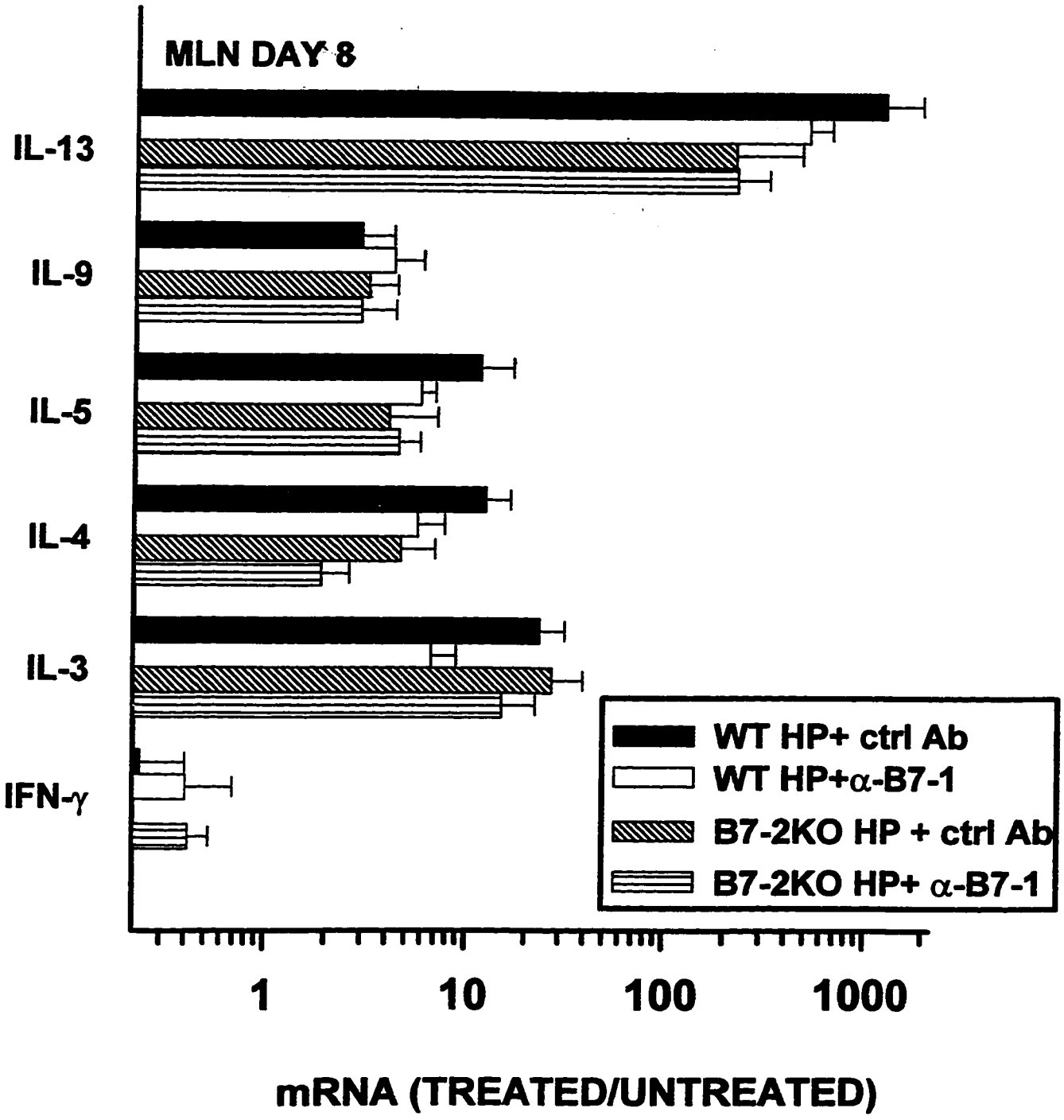

MLN DAY 8
IL-13
IL-9
IL-5
IL-4
IL-3
IFN-γ
WT HP+ ctrl Ab
WT HP+α-B7-1
B7-2KO HP + ctrl Ab
B7-2KO HP+ α-B7-1
1
10
100
1000
mRNA (TREATED/UNTREATED)

Figure 13. At day 14 after *H. polygyrus*-inoculation, elevations in MLN cytokine gene expression are inhibited in B7-2KO but not B7-2WT mice. The mean and standard error derived from individually assayed MLNs of five BALB/c mice are shown for each treatment group. Cytokine gene expression levels were determined using a quantitative RT-PCR as described in Fig. 12. These experiments were repeated two times with similar results.

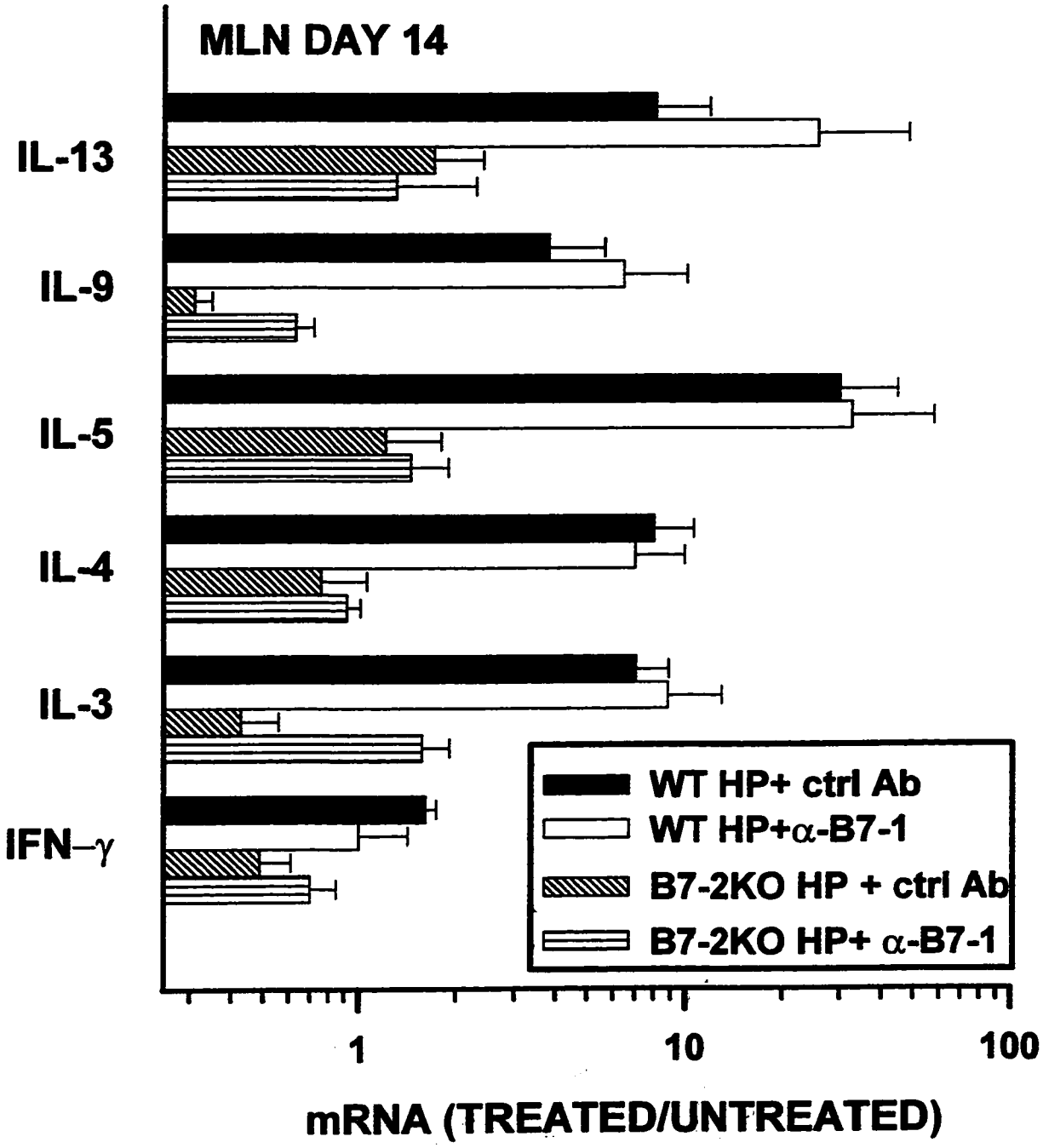
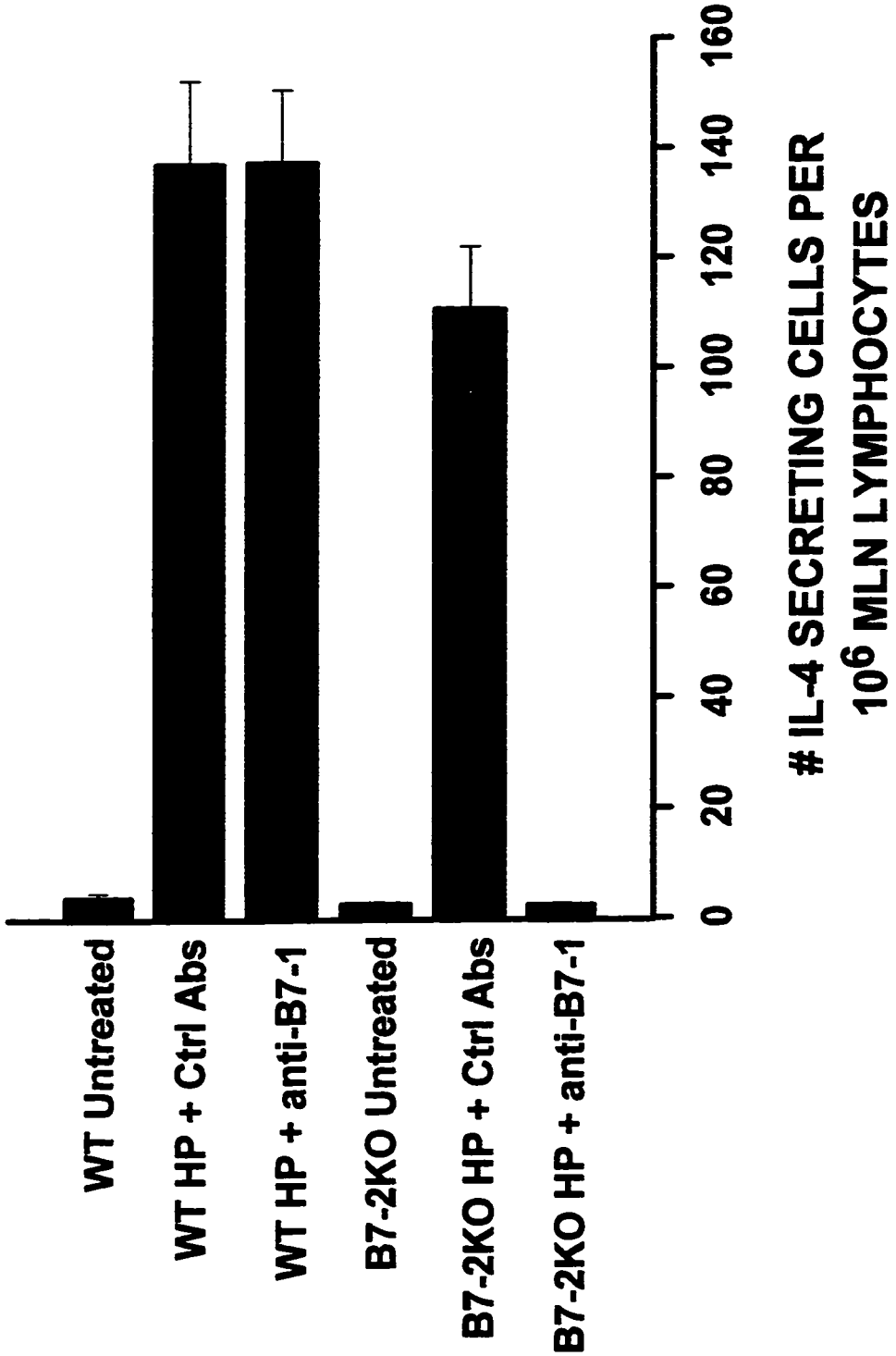

MLN DAY 14
IL-13
IL-9
IL-5
IL-4
IL-3
IFN–γ
WT HP+ ctrl Ab
WT HP+α-B7-1
B7-2KO HP + ctrl Ab
B7-2KO HP+ α-B7-1
1
10
100
mRNA (TREATED/UNTREATED)

Figure 14. B7-2KO and B7-2WT mice exhibit comparable elevations in IL-4 secretion following *H. polygyrus* inoculation. MLN tissues were collected at 8 days after inoculation and the number of IL-4 secreting cells/10^6 MLN cells were determined in an IL-4 specific ELISPOT assay without re-stimulation. The mean and standard error derived from individually assayed MLNs of five BALB/c mice are shown for each treatment group.


WT Untreated
WT HP + Ctrl Abs
WT HP + anti-B7-1
B7-2KO Untreated
B7-2KO HP + Ctrl Abs
B7-2KO HP + anti-B7-1
0
20
40
60
80
100
120
140
160
IL-4 SECRETING CELLS PER
10^6 MLN LYMPHOCYTES

was markedly elevated in *H. polygyrus*-inoculated B7-2WT or B7-2KO mice given control Abs, and *H. polygyrus*-inoculated B7-2WT mice given anti-B7-1 mAbs. However, *H. polygyrus*-inoculated B7-2KO mice administered anti-B7-1 antibodies showed inhibited IL-4 secretion.

The chronic type 2 immune response at day 14 after *H. polygyrus* inoculation is characterized by sustained elevations in cytokine gene expression. Using quantitative RT-PCR analysis, elevations in cytokine mRNA were markedly inhibited in *H. polygyrus*-inoculated B7-2KO as compared to inoculated B7-2WT mice. In particular IL-3, IL-4, IL-5, IL-9, and IL-13 were inhibited to untreated control levels in the MLN and at least partially inhibited in the PP (data not shown), the residual expression perhaps coming from non-T cells. Thus, these findings thus suggest that although B7-2 is not required for the initiation of the T cell cytokine response, B7-2 is necessary for sustaining the type 2 immune response at later stages of the immune response. At these later time points, IL-4 secretion is frequently difficult to detect and consequently could not be assessed by ELISPOT in these experiments.

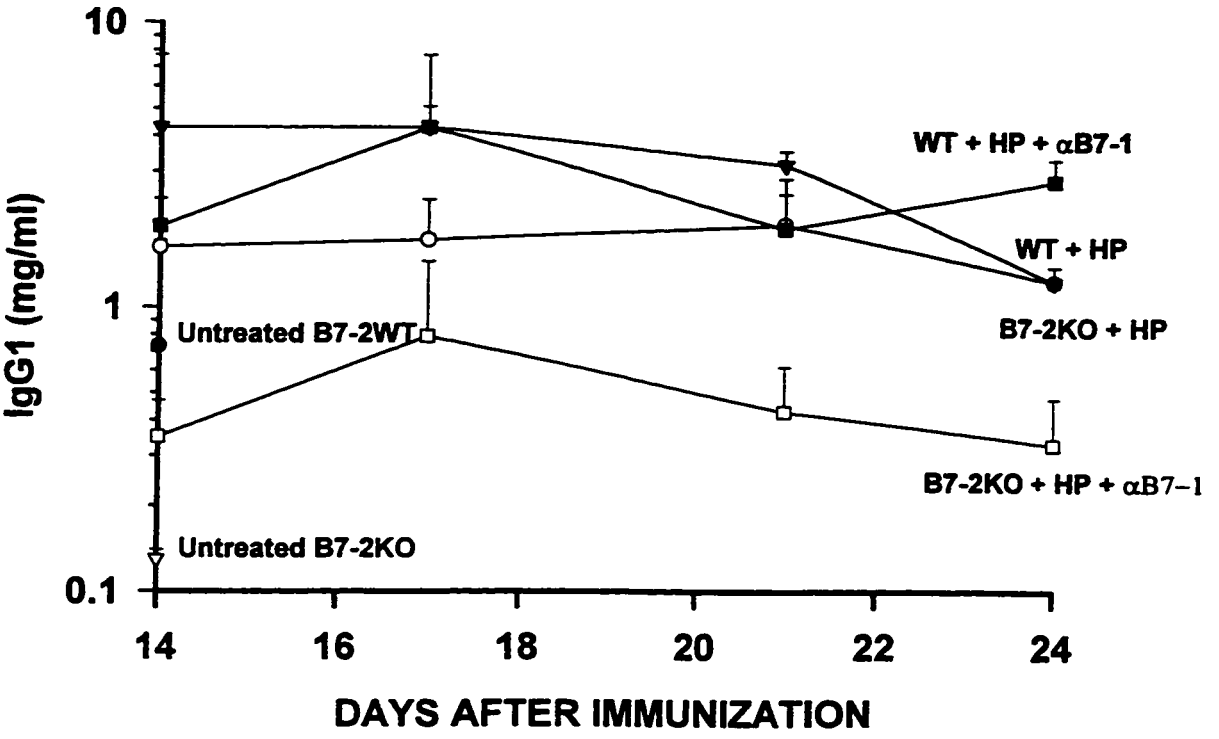
3. Increases in serum IgE are preferentially B7-2-dependent during the *H. polygyrus* Th2 immune response

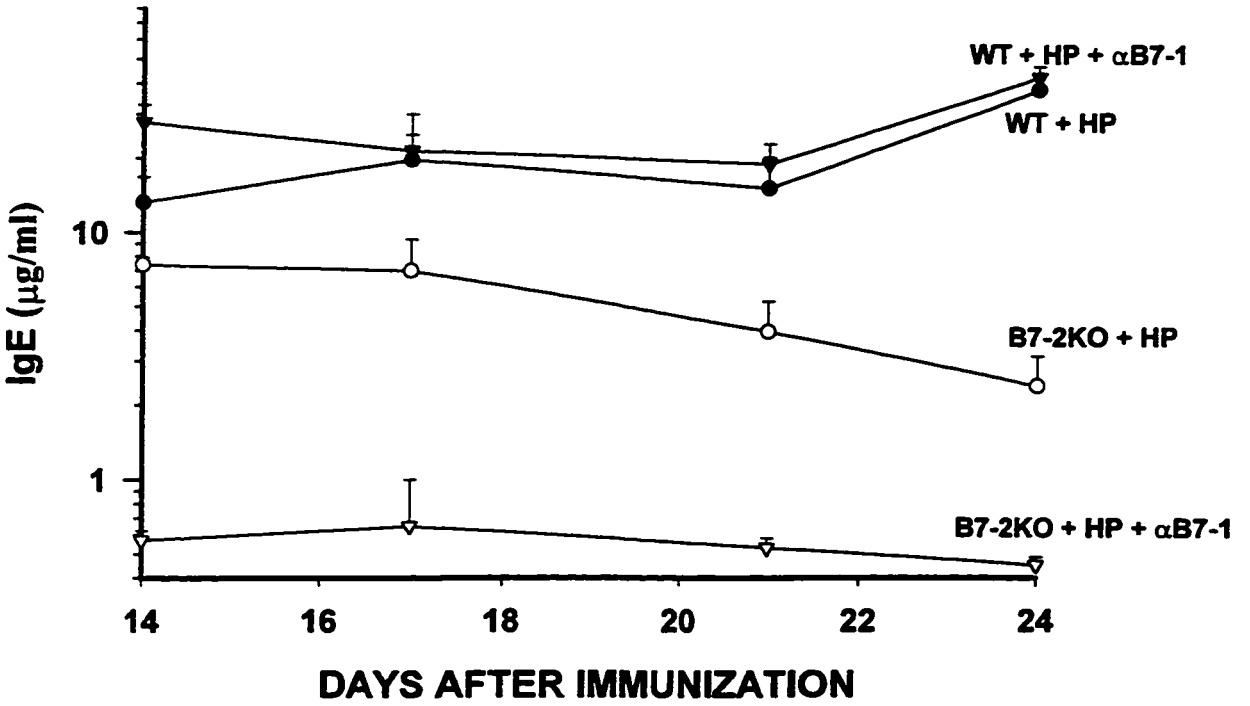
Inhibition of Th2 cytokine gene expression at day 14 after *H. polygyrus* inoculation suggested that T cell effector function was impaired at this late stage of the primary response, although GC formation was sustained. Previous studies have shown that although elevations in both serum IgE and IgG1 are $CD4^+$ T cell-dependent, IgE

elevations are IL-4-dependent while increases in IgG1 are IL-4-independent. To examine whether secretion of these antibody classes differ with regards to B7-2 dependence, serum IgG1 and IgE levels were measured at day 14 in the same experiment where elevations in cytokine gene expression were analyzed. Marked B7-1-dependent serum IgG1 elevations were detected in *H. polygyrus*-inoculated BALB/c and BALB/c B7-2KO mice, while serum IgE elevations were slightly decreased in *H. polygyrus*-inoculated B7-2KO compared to *H. polygyrus*-inoculated BALB/c-B7-2WT at day 14 after inoculation.

To further examine whether elevations in serum IgE were more B7-2-dependent than increases in serum IgG1 at later stages of the response, a separate experiment was conducted where serum Ig levels were assessed at day 14, 17, 21, and 24 after *H. polygyrus* inoculation. As shown in Fig. 15, serum IgE elevations were increasingly different between *H. polygyrus*-inoculated B7-2KO and B7-2WT mice at later stages of the response with an approximately 30-fold difference being detected by day 24. In contrast, serum IgG1 levels differed only approximately 2-fold throughout the course of the response for both *H. polygyrus*-inoculated B7-2KO and B7-2WT mice (Fig. 15). Furthermore, there remained a substantial B7-1-dependent humoral component since anti-B7-1 mAb treatment markedly inhibited increases in both serum IgE and IgG1 in B7-2KO but not B7-2WT *H. polygyrus*-inoculated mice.

Figure 15. Serum IgG1 elevations are comparable in B7-1KO and B7-2WT mice while serum IgE elevations are preferentially inhibited in B7-2KO mice after *H. polygyrus* inoculation. Mice were bled by orbital perfusion and after oral inoculation with 200 third-stage *H. polygyrus* larvae, bled at days 14, 17, 21, and 24 and serum IgG1 and IgE elevations determined using an ELISA. Untreated animals exhibited serum IgG1 levels were less than 1 mg/ml and serum IgE levels were undetectable. The mean and SE derived from five individual BALB/c mice are shown for each timepoint. These experiments were repeated two times with similar results.


10
1
0.1
IgG1 (mg/ml)
WT + HP + αB7-1
WT + HP
B7-2KO + HP
Untreated B7-2WT
B7-2KO + HP + αB7-1
Untreated B7-2KO
14
16
18
20
22
24
DAYS AFTER IMMUNIZATION

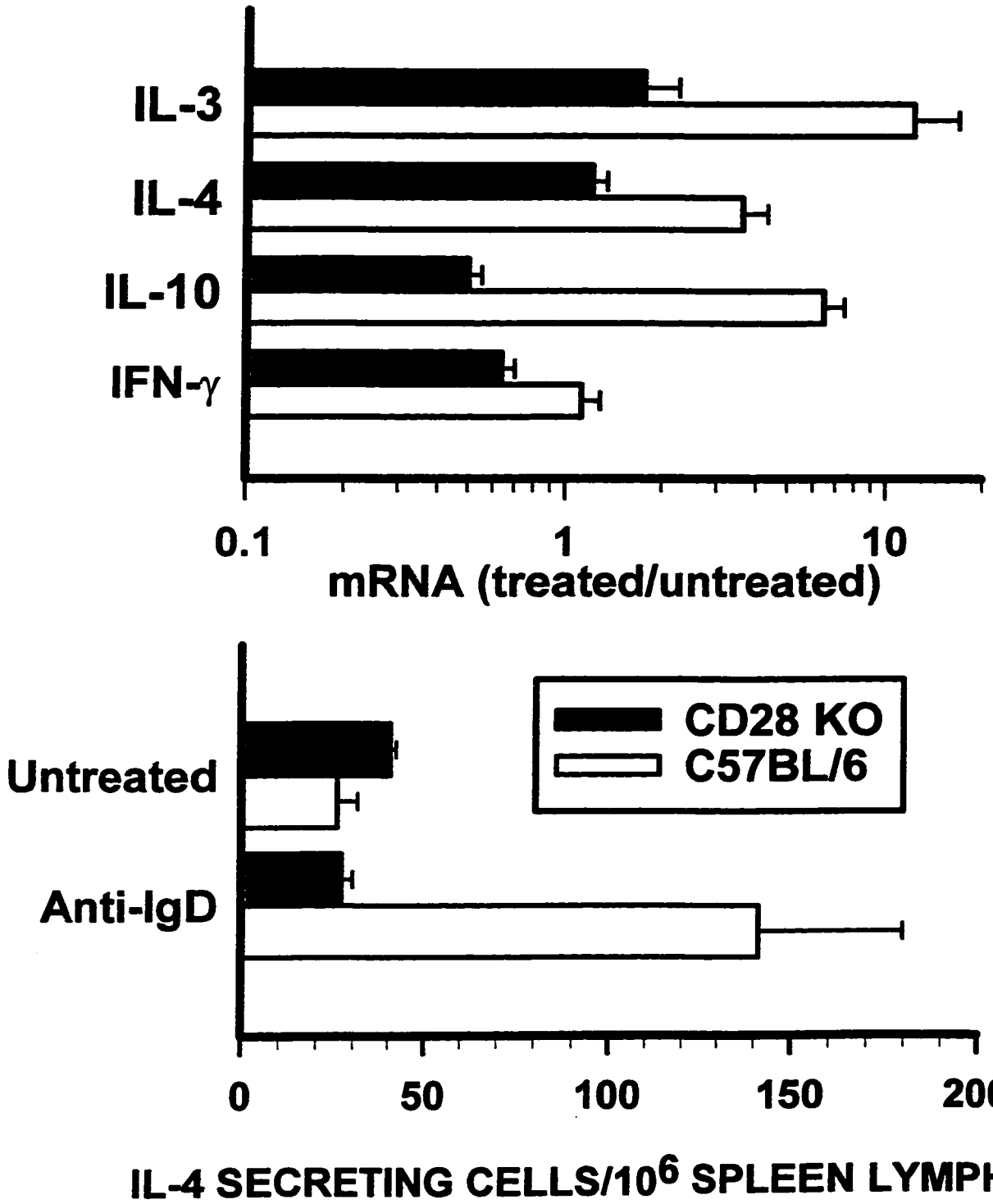

WT + HP + αB7-1
WT + HP
10
1
IgE (μg/ml)
B7-2KO + HP
B7-2KO + HP + αB7-1
14
16
18
20
22
24
DAYS AFTER IMMUNIZATION

C. CD28 dependence of T cell differentiation to IL-4 production varies with the particular type 2 immune response

1. Anti-IgD-induced elevations in cytokine expression are blocked in CD28KO mice

Previous studies showed that T cell-derived cytokine gene expression, as detected by a quantitative RT-PCR assay, included marked elevations in IL-3, IL-4, and IL-10 mRNA on day 6 after GAMδ immunization (Svetic et al., 1991) and that these increases in cytokine mRNA levels were blocked by the administered of CTLA-4Ig fusion protein (Lu et al., 1995). These findings suggested that B7 ligands, CD28 and CTLA-4, were required for the development of cytokine-producing T cells. To determine whether CD28 was providing the required costimulatory signal for the development of cytokine-producing T cells, C57BL/6 CD28WT and CD28KO mice (four to five per treatment group) were immunized i.v. with 800 μgs GaMδ, and six days later, elevations in spleen cytokine gene expression and IL-4 secretion were determined. As shown in Fig. 16, marked elevations in IL-3, IL-4 and IL-10 mRNA were detected in CD28WT mice. In contrast, elevations in these cytokines were not detected in CD28KO mice. The inhibition of cytokine elevations in CD28KO mice is consistent with results from CTLA-4Ig treated anti-IgD immunized mice, in which elevations in IL-10, as well as IL-4, were blocked (Lu et al., 1995). In contrast, elevations in IL-4, but not IL-10, were blocked in anti-IgD-immunized BALB/c mice (Lu et al., 1995).

Figure 16. Elevations in cytokine gene expression and IL-4 protein secretion are blocked in CD28-deficient mice on day 6 after anti-mouse IgD Ab immunization. CD28KO or CD28WT mice (five per treatment group) were injected i.v. with 800μg of GaMδ. The mean and SE from spleens of individual mice are shown. A quantitative RT-PCR assay was used to measure relative differences in cytokine gene expression. All data, quantitated using a Phosphoimager (Molecular Dynamics), were individually normalized to the housekeeping gene, HPRT, which did not change by more than two- or threefold throughout the experiment. The means are expressed relative to the mean of the untreated control group, which was arbitrarily given a value of 1. The number of IL-4 secreting cells per 10^6 was determined in an ELISPOT assay in which cells were cultured for only 3 hours without restimulation.

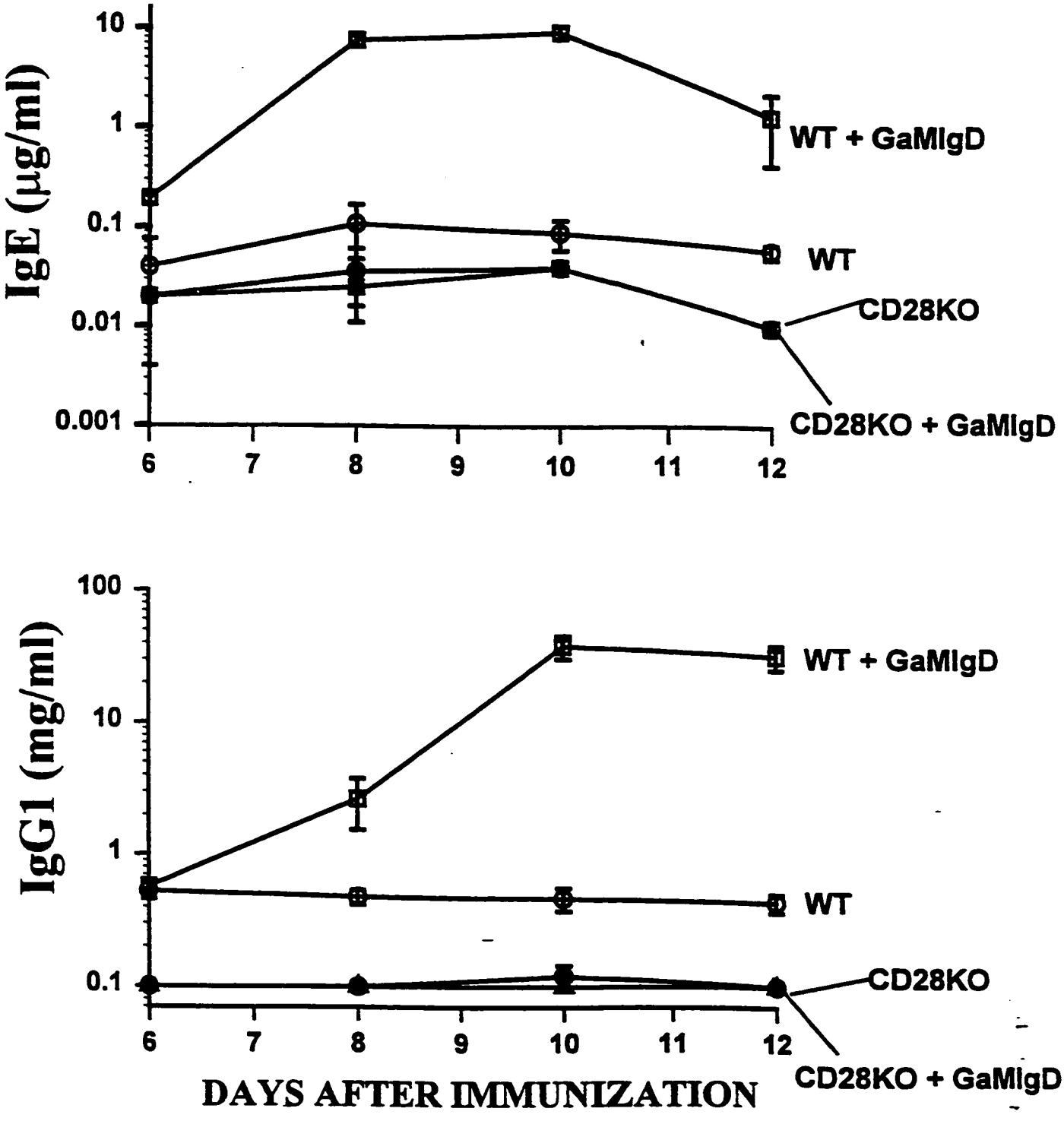

IL-3
IL-4
IL-10
IFN-γ
0.1
1
10
mRNA (treated/untreated)
CD28 KO
C57BL/6
Untreated
Anti-IgD
0
50
100
150
200
IL-4 SECRETING CELLS/10^6 SPLEEN LYMPHOCYTES

To determine whether the secretion of IL-4, a primary T cell-derived cytokine regulating the development of the type 2 immune response, was also absent in CD28KO mice, an ELISPOT assay was performed to assess the number of IL-4-secreting spleen cells from anti-IgD immunized mice. This assay is a considerable improvement over other *in vitro* assays that require extended cultures, in that cells are cultured in vitro for only 3 hours without mitogens. The results shown in Fig. 16 show that the number of IL-4 secreting cells was increased five-fold in GaMδ-immunized mice but was not elevated over the untreated control value in GaMδ-immunized CD28KO mice.

2. Serum Ig levels are not elevated in anti-IgD-immunized CD28-deficient mice

Previous results have shown substantial increases in serum IgE and IgG1 by 8 to 10 days after immunization with GaMδ Abs (Finkelman et al., 1990). In addition, elevations in serum Ig levels were blocked in anti-IgD-immunized mice given CTLA-4Ig on days 0 and 1 after immunization (Lu et al., 1995). To examine whether CD28 was required for serum IgE and IgG1 elevations following GaMδ immunization, CD28WT and CD28KO mice were injected i.v. with 800μgs of GaMδ Abs and evaluated for serum IgG1 and IgE elevations on days 6, 8, 10 and 12 after immunization. Although both IgG1 and IgE levels were increased approximately 100-fold by day 10 after immunization of CD28WT mice, no increases in either serum IgG1 or IgE were detected in GaMδ Ab-immunized CD28KO mice (Fig. 17).

Figure 17. Elevations in serum IgG1 and IgE were blocked in CD28-deficient mice after GaMδ immunization. CD28KO or CD28WT (five per treatment group) were bled by orbital perfusion and injected i.v. with 800μg of GaMδ Ab. Serum IgG1 and IgE levels were measured on days 6, 8, 10, and 12 after immunization by ELISA.


IgE (µg/ml)
10
1
0.1
0.01
0.001
6
7
8
9
10
11
12
WT + GaMIgD
WT
CD28KO
CD28KO + GaMIgD
IgG1 (mg/ml)
100
10
1
0.1
WT + GaMIgD
WT
CD28KO
CD28KO + GaMIgD
DAYS AFTER IMMUNIZATION

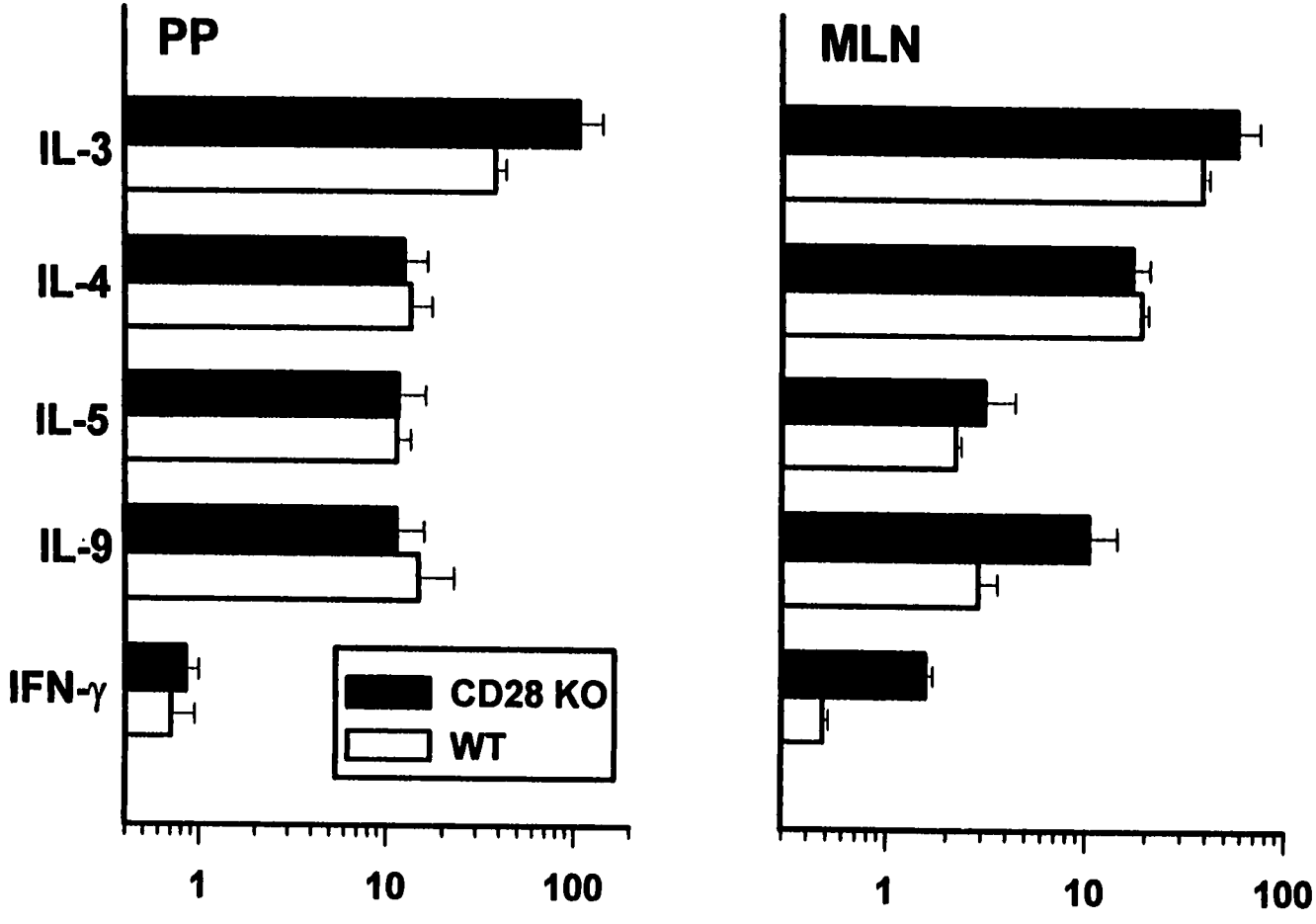
3. *H. polygyrus*-induced cytokine mRNA gene expression is comparable in CD28WT and CD28KO mice.

Previous studies have demonstrated that by day 8 after *H. polygyrus* inoculation, elevations in IL-3 and IL-5 are at least partly $CD4^+$ T cell dependent in Peyer's patch (PP) and MLN, and that elevations in IL-4 are derived exclusively from TCR-$\alpha\beta^+$, $CD4^+$ T cells. Furthermore, blocking CTLA-4 ligand interactions during the *in vivo* immune response to *H. polygyrus* completely blocked elevations in IL-4 and partially blocked elevations in IL-3 and IL-5 gene expression in the PP and MLN (Lu et al., 1994). To determine whether cytokine elevations were blocked in *H. polygyrus*-inoculated CD28KO mice, CD28WT and CD28KO mice were orally inoculated with third-stage *H. polygyrus* larvae. Eight days after inoculation, when cytokine levels peak during this response, PP and MLN were removed from the mice (five per treatment group) and individually analyzed for cytokine gene expression. Elevations in IL-3, IL-4, IL-5 and IL-9 cytokine gene expression were comparable in CD28WT and CD28KO mice (Fig. 18). Also, IFN-γ levels remained comparable to untreated control values in *H. polygyrus*-inoculated CD28KO mice, suggesting that immune deviation did not occur.

4. $CD4^+$, TCR$\alpha\beta^+$ T cells are the primary source of elevated IL-4 in CD28KO mice after *H. polygyrus* inoculation

In normal mice, the primary source of elevated IL-4 in *H. polygyrus*-inoculated mice is derived from $CD4^+$, TCR$\alpha\beta^+$ T cells. To investigate whether this was also the

Figure 18. Elevations in cytokine gene expression following *H. polygyrus* inoculation are comparable in CD28-deficient mice and normal controls. CD28KO and CD28WT mice (five mice per treatment group) were orally inoculated with 200 *H. polygyrus* larvae; eight days later, MLN tissues were collected, and cytokine gene expression levels were determined by a quantitative RT-PCR assay, as described in Fig. 16. The mean and SE for each treatment group are shown. This experiment was repeated twice with similar results.

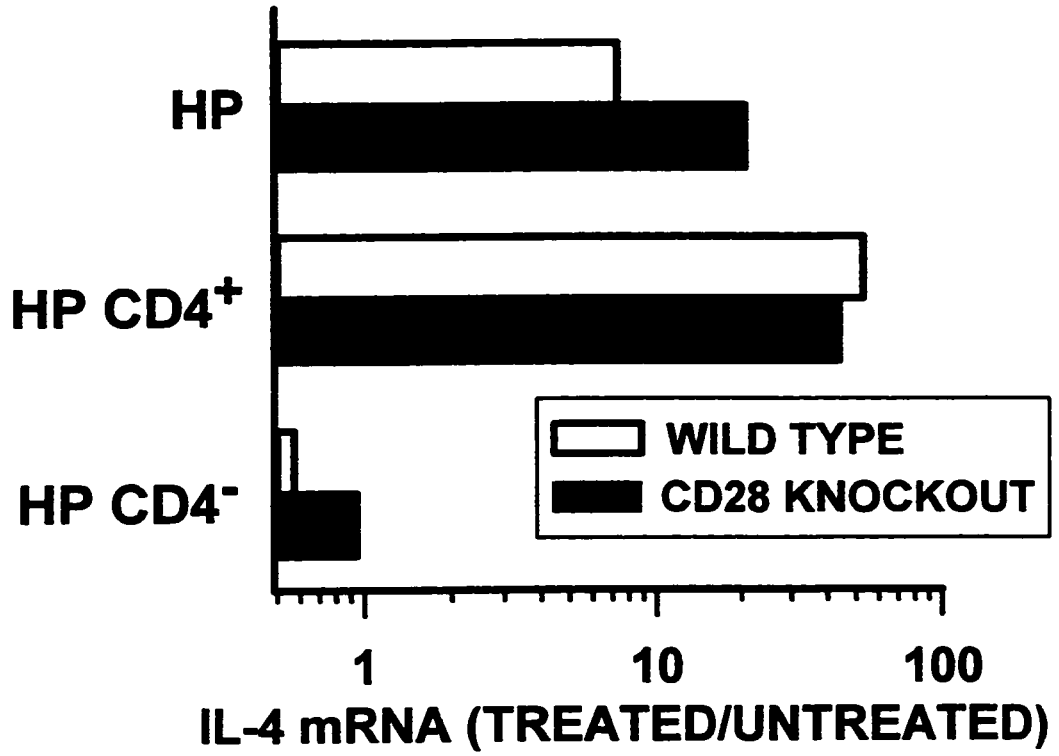

PP
MLN
IL-3
IL-4
IL-5
IL-9
IFN-γ
CD28 KO
WT
1
10
100
1
10
100
FACTOR INCREASE OVER UNTREATED

case in CD28KO mice or that IL-4 was produced by non-T cells resulting from changes due to the gene knockout, on day 8 after *H. polygyrus* inoculation, pooled MLN cell suspensions from *H. polygyrus*-inoculated CD28WT and CD28KO mice were sorted by FACS into suspensions (five mice per treatment group) of TCR$\alpha\beta^+$, $CD4^+$ and non-TCR$\alpha\beta^+$, $CD4^+$ T cells. As shown in Fig. 19, in *H. polygyrus*-inoculated mice, the primary sources of elevations in IL-4 gene expression and protein secretion were TCR$\alpha\beta^+$, $CD4^+$ T cells in CD28KO as well as CD28WT mice. These findings confirm that $CD4^+$ T cells can differentiate to produce IL-4 in *H. polygyrus*-inoculated mice deficient in CD28, demonstrating that a non-T cell was not the source of elevated IL-4 in the CD28KO mice.

5. Elevated serum Ig levels in *H. polygyrus*-inoculated CD28KO mice

As in the anti-IgD immune response, marked elevations in serum IgE and IgG1 levels occurred following oral inoculation with *H. polygyrus* (Finkelman et al., 1990). To examine whether CD28 deficiency also blocked serum Ig elevations in the *H. polygyrus* system, CD28WT and CD28KO mice were orally inoculated with 200 third-stage *H. polygyrus* larvae, and on days 10, 12, 14 and 16 after inoculation serum IgE and IgG1 levels are evaluated. As shown in Fig. 20, significant increases in serum IgE and IgG1 levels were detected in CD28KO as well as CD28WT mice inoculated with *H. polygyrus*. In a separate experiment, serum IgG2a levels were also measured on day 14 after *H.*

Figure 19. $CD4^+$, $TCR\alpha\beta^+$ cells express IL-4 mRNA and secrete IL-4 protein in CD28-deficient mice immunized with *H. polygyrus*. MLN cells from *H. polygyrus*-inoculated CD28WT and CD28KO mice (five per treatment group) were collected on day 8 after immunization. $CD4^+$, $TCR\alpha\beta^+$ and non- $CD4^+$, $TCR\alpha\beta^+$ MLN cells were sorted on the same day using an EPICS Elite cell sorter and were shown to be greater than 97% pure. The sorted cell populations were used to determine IL-4 mRNA expression by RT-PCR and IL-4 secretion by the ELISPOT assay.


HP
HP CD4+
HP CD4-
WILD TYPE
CD28 KNOCKOUT
1
10
100
IL-4 mRNA (TREATED/UNTREATED)

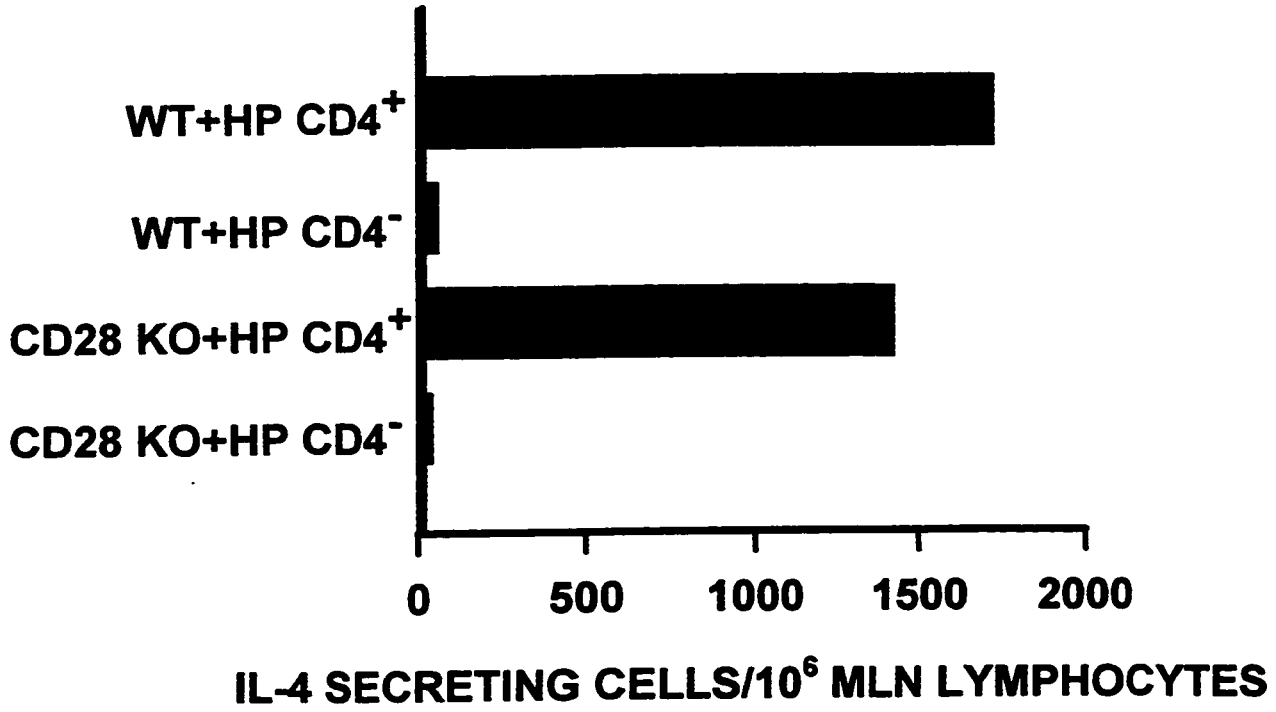
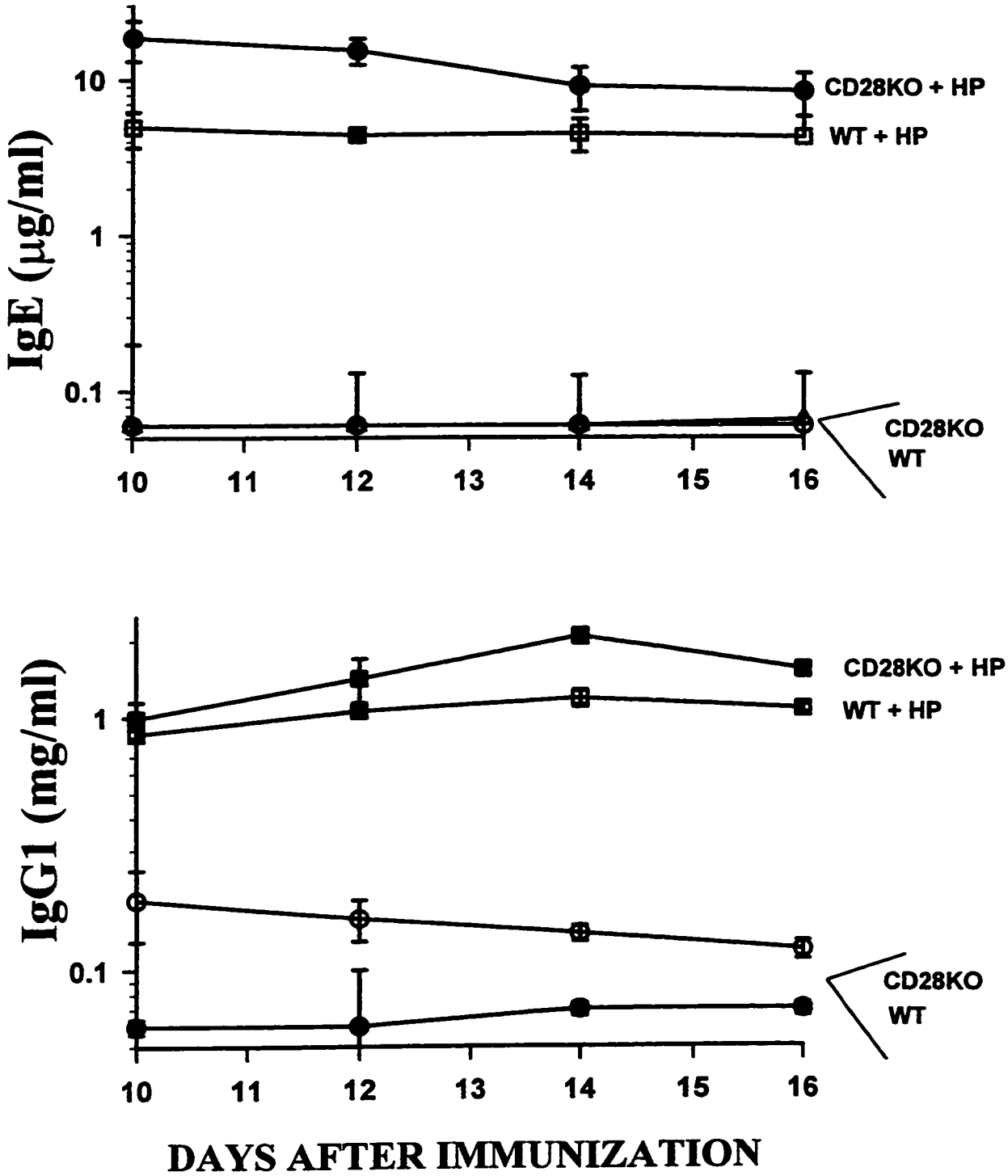

WT+HP CD4+
WT+HP CD4-
CD28 KO+HP CD4+
CD28 KO+HP CD4-
0
500
1000
1500
2000
IL-4 SECRETING CELLS/10^6 MLN LYMPHOCYTES

Figure 20. Serum IgG1 and IgE levels are elevated in *H. polygyrus*-inoculated CD28-deficient mice. Serum Ig levels were collected by orbital perfusion and measured on days 10, 12, 14, and 16 after oral inoculation with 200 *H. polygyrus* larvae to CD28KO or CD28WT mice (five per treatment group). Serum IgE and IgG1 levels were detected by ELISA. The mean and SE are shown for individual animals (four-five per treatment group). This experiment was repeated twice with similar results.


IgE (µg/ml)
10
1
0.1
CD28KO + HP
WT + HP
CD28KO
WT
10
11
12
13
14
15
16
IgG1 (mg/ml)
1
0.1
CD28KO + HP
WT + HP
CD28KO
WT
10
11
12
13
14
15
16
DAYS AFTER IMMUNIZATION

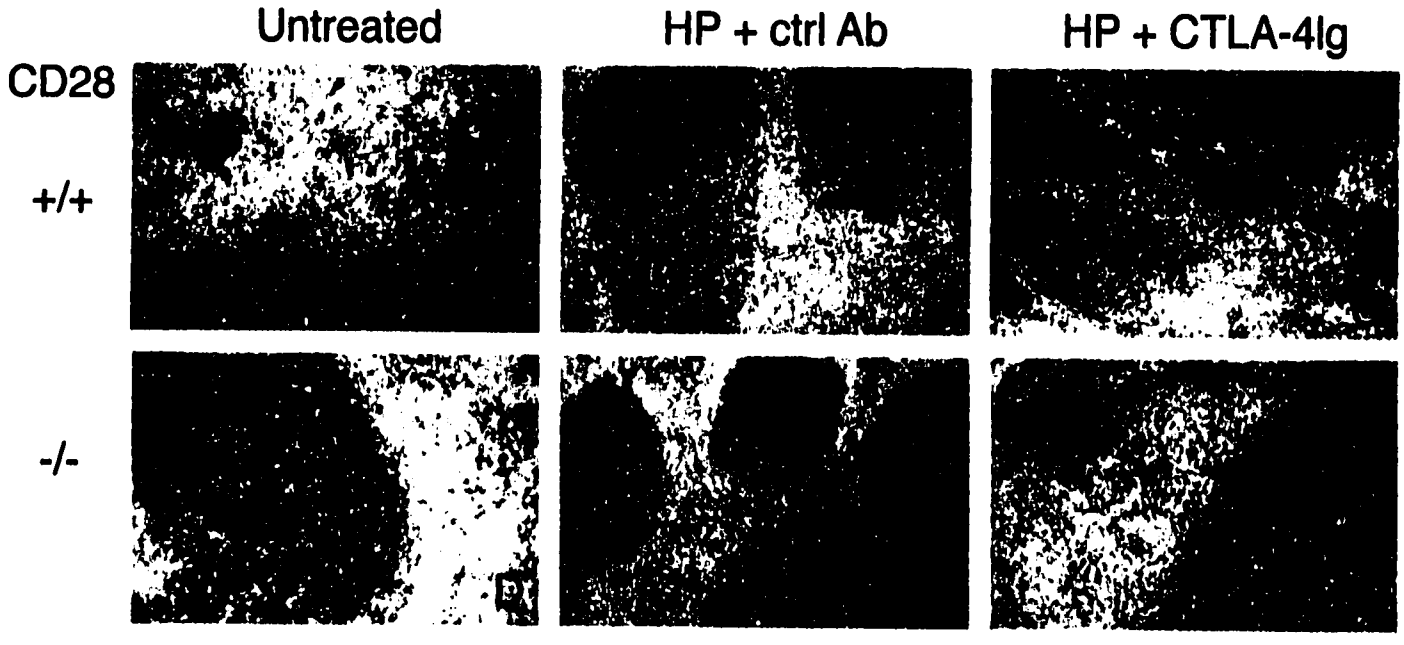
polygyrus inoculation and significant increases were not detected in either CD28KO or CD28WT mice.

D. Studies of CTLA-4Ig Administration in CD28KO mice

1. GC formation is comparable in *H. polygyrus*-inoculated CD28WT and CD28KO mice and blocked by CTLA-4Ig administration

Recent studies have suggested that CD28 interactions may be required for GC formation (Lane et al., 1994; Ferguson et al., 1996). To examine whether CD28 is required for GC formation during the type 2 immune response to *H. polygyrus*, MLN tissues were collected from CD28WT and CD28KO mice at day 14 following oral inoculation of 200 third-stage *H. polygyrus* larvae. GC volume measurements were comparable in CD28WT and CD28KO mice after immunization, although overall lymph node size was reduced in CD28KO mice (data not shown). CTLA-4Ig administration blocked GC formation after *H. polygyrus* inoculation in both CD28WT and CD28KO mice (Fig. 21). These results suggest that a B7 ligand besides CD28 can provide the costimulatory signal required for the development of GCs. Since CTLA-4Ig blocks GC formation in this response and CTLA-4 and CD28 are the only known B7 ligands, our studies suggest that CTLA-4 may provide the initial costimulatory signal in the absence of CD28.

Figure 21. CTLA-4Ig administration to *H. polygyrus*-inoculated CD28KO mice blocks elevations in GC formation. MLN tissues were stained at day 8 for $CD4^+$ T cells with GK1.5 (blue stain) and GC cells with the lectin, PNA (red stain), as described in *Materials and Methods*. (A-F) CD28KO mice (4/treatment group) were orally inoculated with 200 third-stage *H. polygyrus* larvae and either i.v. administered 200 μg of CTLA-4Ig or the control fusion protein, L6 on days 0 and 1. GC formation was pronounced in *H. polygyrus*-inoculated CD28WT and CD28KO mice given L6, but was blocked in *H. polygyrus*-inoculated CD28WT and CD28KO mice given CTLA-4Ig.


Untreated
HP + ctrl Ab
HP + CTLA-4Ig
CD28
+/+
-/-

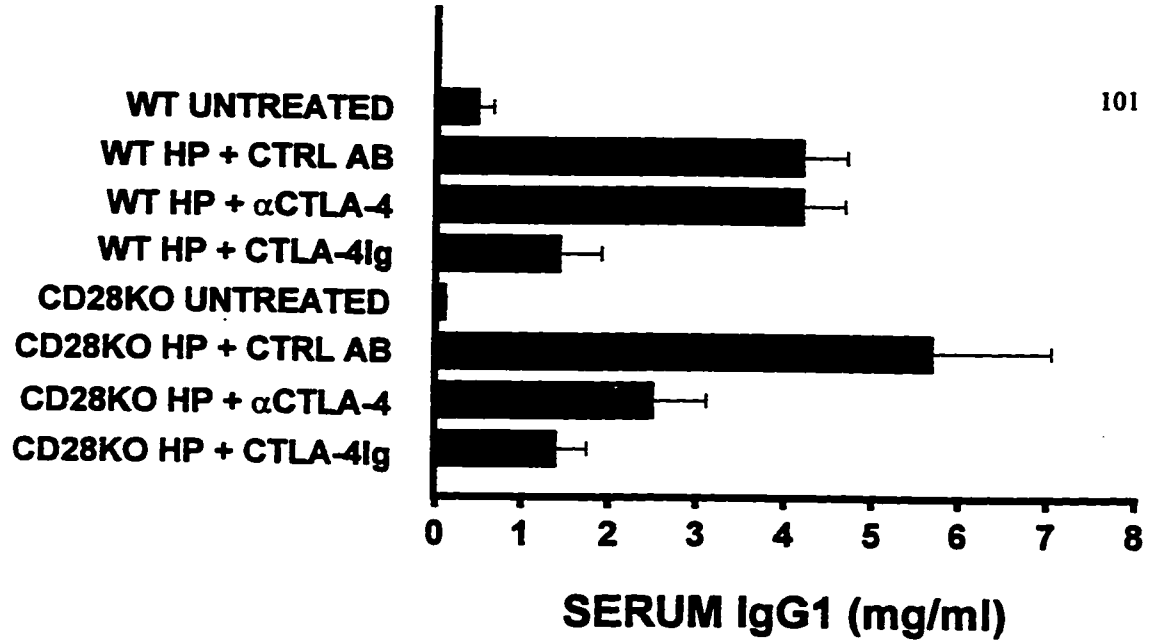
2. Elevations in serum IgG1 and IgE levels are blocked in *H. polygyrus*-inoculated in CD28KO mice administered CTLA-4Ig.

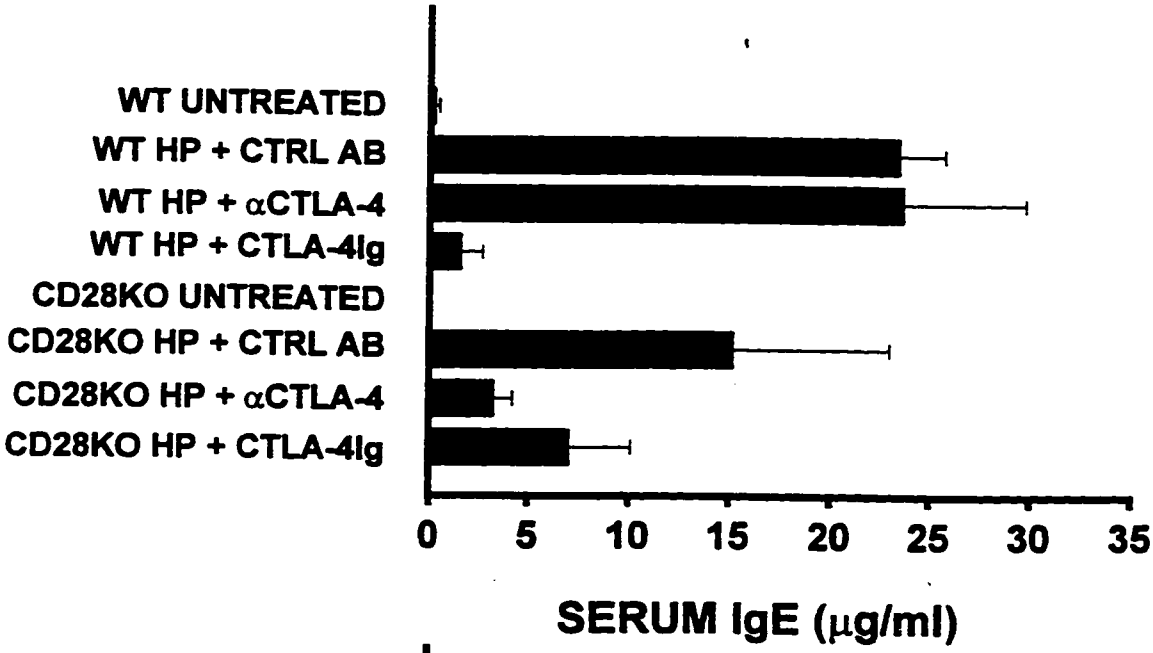
Our findings that B7-dependent GC formation following *H. polygyrus* inoculation CTLA-4Ig administration was blocked in CD28KO suggested that elevations in serum IgG1 and IgE levels may also be affected. As shown in Fig. 22, significant increases in serum IgG1 levels were detected in *H. polygyrus*-inoculated CD28KO mice administered control antibodies. In contrast, elevations in serum IgG1 were blocked in *H. polygyrus*-inoculated CD28KO mice administered anti-B7-1 mAb and CTLA-4Ig, respectively.

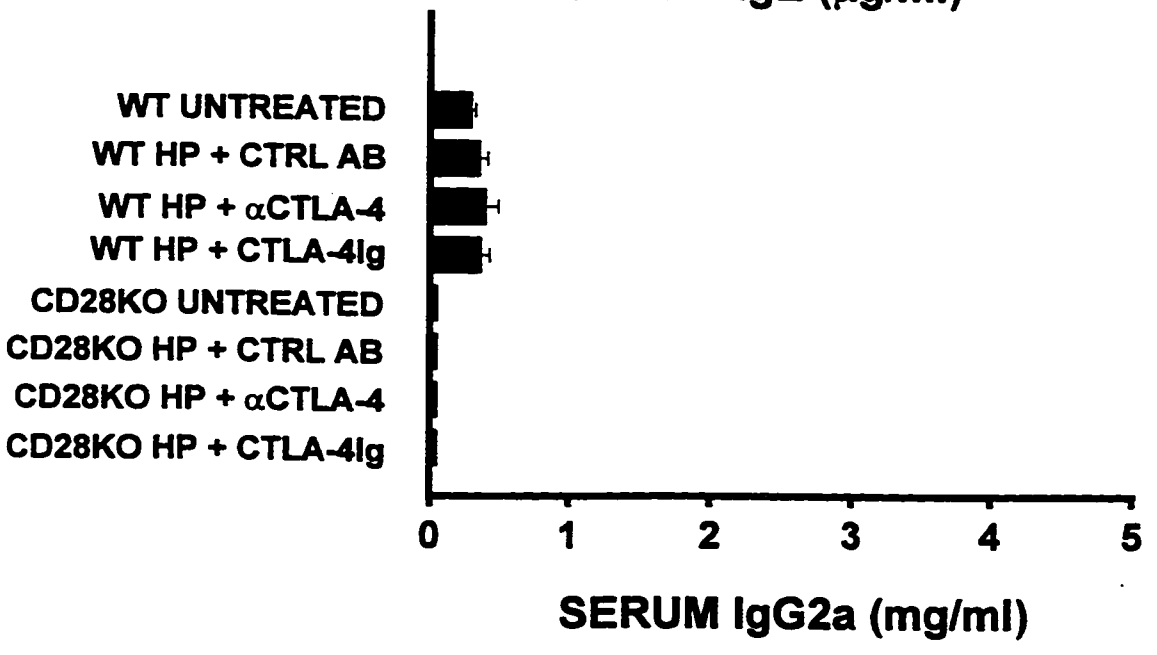
3. T cell derived IL-4 production varies in *H. polygyrus*-inoculated CD28KO mice administered CTLA-4Ig.

In several experiments, our studies have shown the inhibition of T cell IL-4 production which is consistent with our GC and serum IgE and IgG1 findings. However, in recent experiments, *H. polygyrus*-inoculated CD28KO mice administered CTLA-4Ig exhibit T cell IL-4 production comparable to control levels (Fig. 23). The possibility exists that there are strain differences in the CD28KO mice used in these experiments or that the mice are infected with another pathogen, and our results are indicating a CD28-independent memory response. We are currently investigating these possibilities.

Figure 22. Elevations in serum IgE and IgG1 levels are inhibited following CTLA-4Ig administration in *H. polygyrus*-inoculated CD28KO mice. CD28WT and CD28KO mice were injected i.v. with 200 μg of CTLA-4Ig or the control fusion protein, L6 on days 0 and 1 after inoculation (4 mice/group). Mice were bled by orbital perfusion at day 14 after inoculation and serum IgE, IgG1 and IgG2a levels were measured on day 14 following immunization by ELISA. The mean and SE derived are shown for each treatment group. This experiment was repeated twice with similar results.


WT UNTREATED
WT HP + CTRL AB
WT HP + αCTLA-4
WT HP + CTLA-4Ig
CD28KO UNTREATED
CD28KO HP + CTRL AB
CD28KO HP + αCTLA-4
CD28KO HP + CTLA-4Ig
0 1 2 3 4 5 6 7 8
SERUM IgG1 (mg/ml)


WT UNTREATED
WT HP + CTRL AB
WT HP + αCTLA-4
WT HP + CTLA-4Ig
CD28KO UNTREATED
CD28KO HP + CTRL AB
CD28KO HP + αCTLA-4
CD28KO HP + CTLA-4Ig
0 5 10 15 20 25 30 35
SERUM IgE (μg/ml)


WT UNTREATED
WT HP + CTRL AB
WT HP + αCTLA-4
WT HP + CTLA-4Ig
CD28KO UNTREATED
CD28KO HP + CTRL AB
CD28KO HP + αCTLA-4
CD28KO HP + CTLA-4Ig
0 1 2 3 4 5
SERUM IgG2a (mg/ml)

E. CD28 and CTLA-4 are required for the initial development of the *in vivo* type 2 immune response in BALB/c mice.

1. Administration of either anti-CD28 or anti-CTLA-4 Abs markedly reduces GC formation and *in situ* expansion of $CD4^+$ T cell populations.

We have shown that the type 2 immune response to *H. polygyrus* is not blocked in CD28 deficient mice (Figs. 19, 23). The recent availability of an anti-CD28 mAb that inhibits *in vivo* CD28 interactions (Perez et al., 1997) allowed us to examine whether the wild-type mice could also utilize other T cell costimulatory pathways in the absence of CD28 interactions. In *H. polygyrus*-inoculated mice administered control Abs, well-developed PNA-positive GCs , as well as markedly expanded $CD4^+$ T cell populations, were detected throughout the T cell rich MLN cortex (Fig. 24B). Administration of anti-CD28 or anti-CTLA-4 mAbs to *H. polygyrus*-inoculated mice reduced GC formation (Fig. 24C, D) as compared to inoculated controls. The combined administration of both anti-CD28 and anti-CTLA-4 mAbs or CTLA-4Ig administration inhibited GC formation and the *in situ* expansion of $CD4^+$ T cell populations (Figs. 24E, F). These results indicate that both CD28 and CTLA-4 can provide the costimulatory signals necessary for increased GC formation and $CD4^+$ T cell expansion in the MLN following *H. polygyrus* inoculation. Similar results were observed in two independent experiments.

GC formation was quantitated in the MLN of *H. polygyrus*-inoculated mice administered 300 μg of anti-CD28 (days 0 and 3) and/or 450 μg and 200 μg of anti-CTLA-4 mAbs

Figure 23. Elevations in T cell derived IL-4 protein secretion are not blocked following *H. polygyrus*-inoculation of CD28KO mice administered CTLA-4Ig. CD28KO mice were injected i.v. with 100 μg of CTLA-4Ig or the control fusion protein, L6 (4 mice/group) on days 0 and 1. Tissues were collected on day 8 after inoculation and the number of IL-4 secreting cells per 10^6 MLN cells was determined by an ELISPOT assay without re-stimulation. The mean and SE are shown for five individual mice per treatment group.

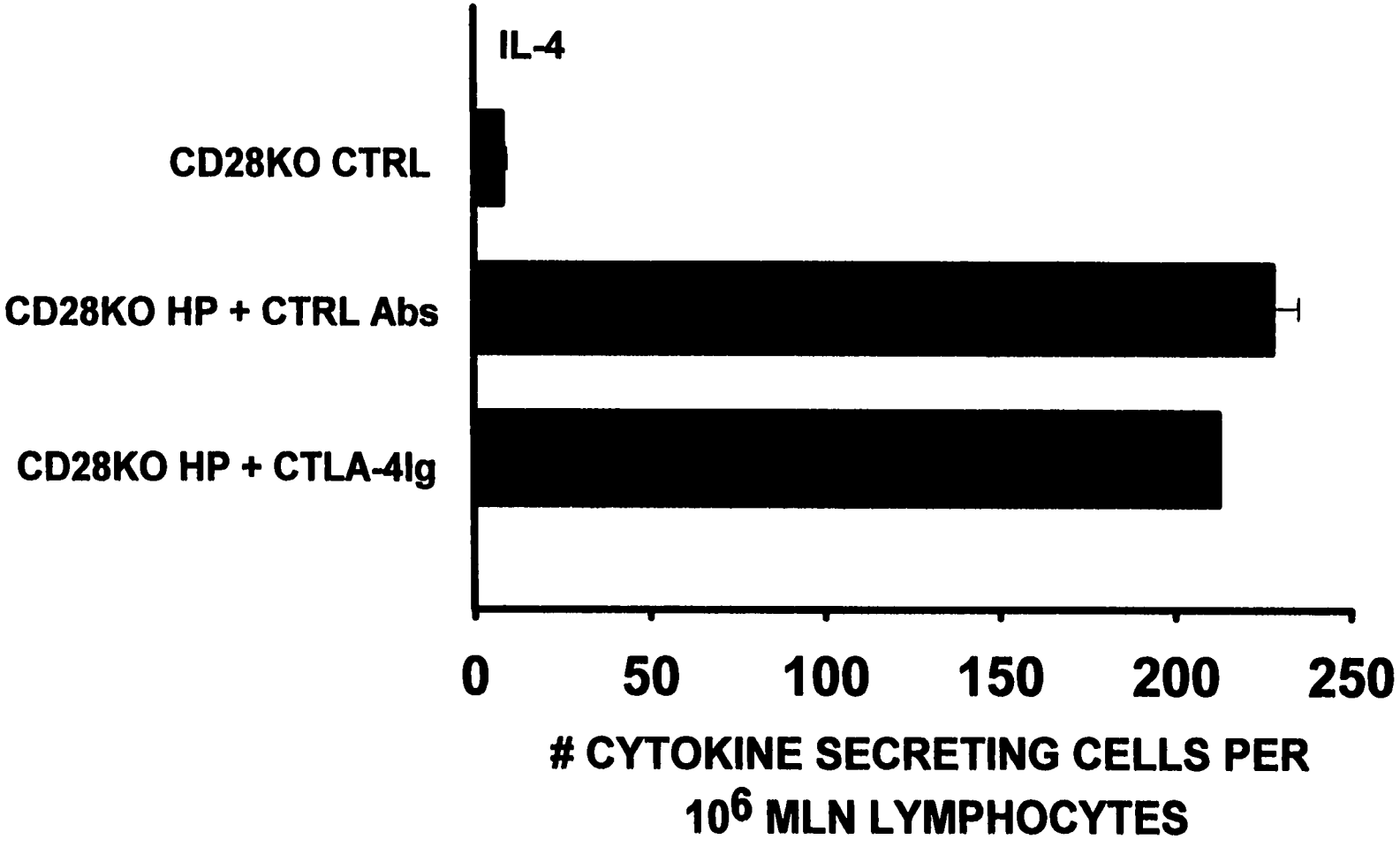
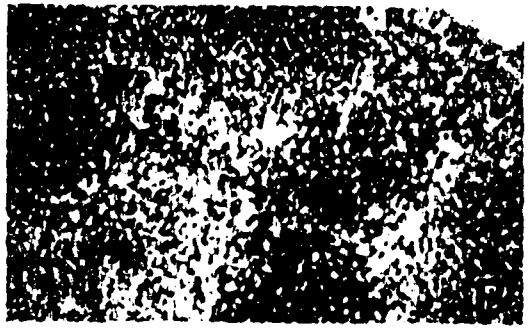

IL-4
CD28KO CTRL
CD28KO HP + CTRL Abs
CD28KO HP + CTLA-4Ig
0
50
100
150
200
250
CYTOKINE SECRETING CELLS PER
10^6 MLN LYMPHOCYTES

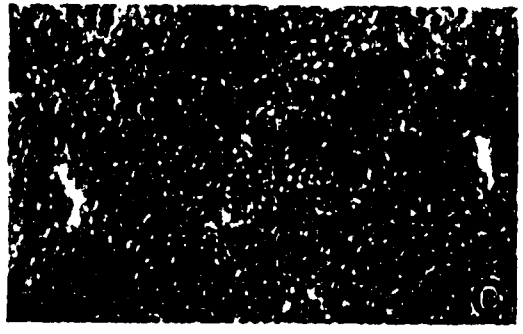
Figure 24. GC formation is reduced in *H. polygyrus*-inoculated WT mice administered anti-CD28 and/or anti-CTLA-4 Abs (C, D). 300μg of anti-CD28 Abs was administered on days 0 and 3 and/or 450μg and 200μg of anti-CTLA-4 Abs were given i.v. on days 0 and 3, respectively. MLN tissues (five mice/group) were collected on day 8 from BALB/c mice, frozen in liquid nitrogen, and subsequently stained at 8μm. Tissue sections were stained for $CD4^+$ T cells with L3T4 (blue stain) and GC cells with PNA (red stain). GC formation was pronounced in *H. polygyrus*-inoculated WT mice (B), but was reduced in *H. polygyrus*-inoculated WT mice given anti-CD28 or anti-CTLA-4 mAbs (C, D). GC formation was completely blocked in inoculated mice administered both anti-CD28 and anti-CTLA-4 Abs (E) or CTLA-4Ig (F).

WT Untreated

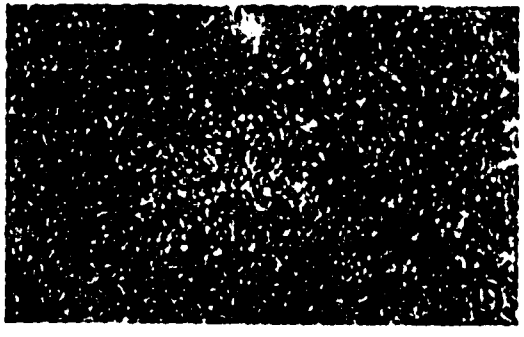
WT HP + ctrl Ab



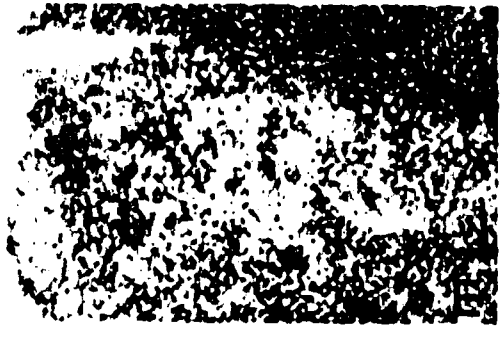
WT HP + anti-CD28



WT HP + anti-CTLA-4 Ab



WT HP + anti-CD-28
+ anti-CTLA-4 Abs



WT HP + CTLA-4Ig

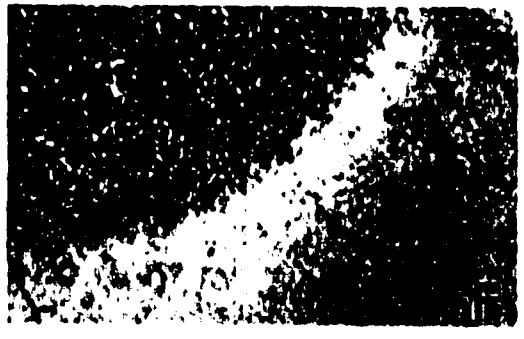
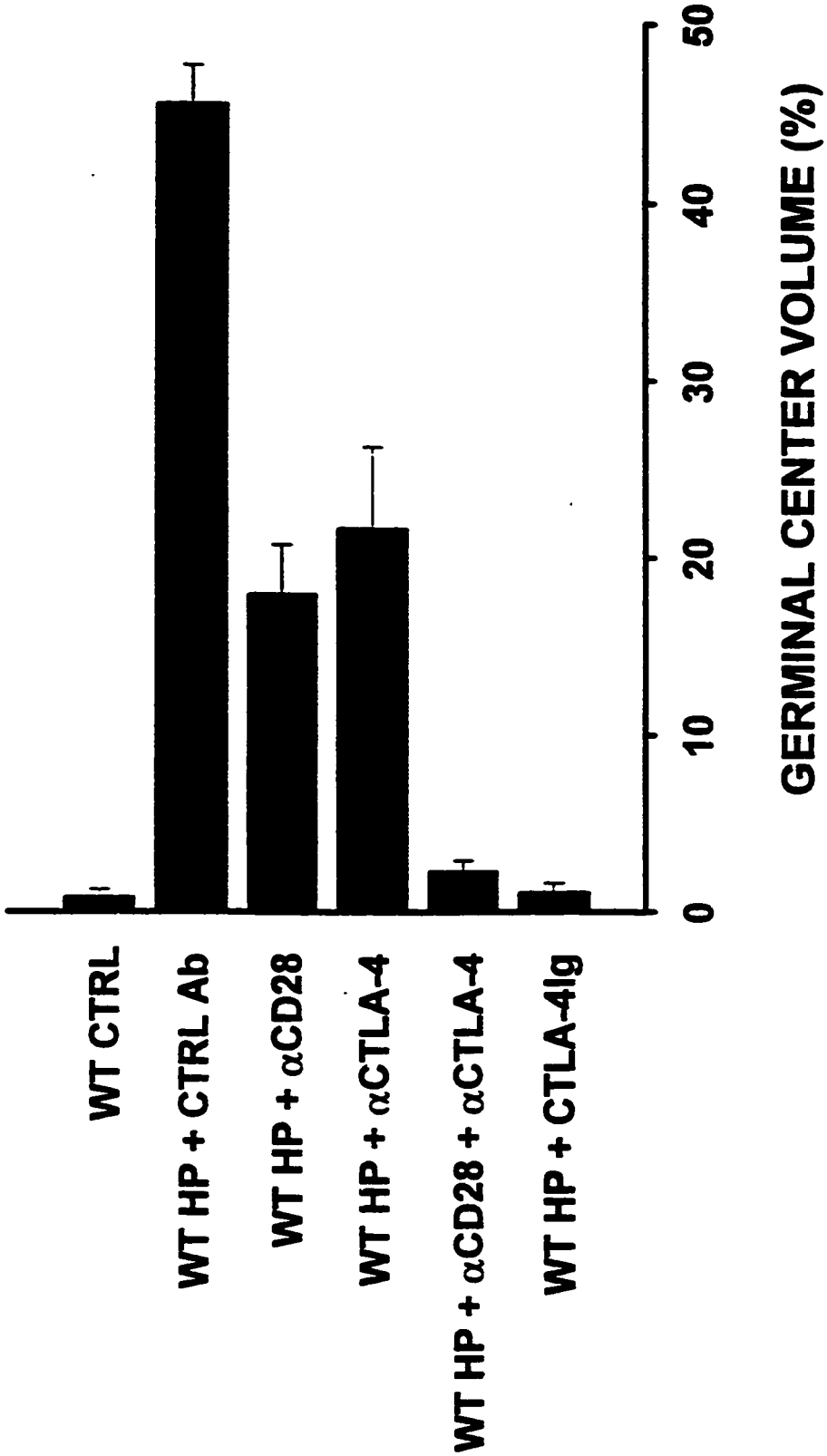


Figure 25. Administration of anti-CD28 or anti-CTLA-4 mAbs to *H. polygyrus*-inoculated wild-type mice reduces the formation of GCs. BALB/c wild-type mice (5/treatment group) were orally inoculated with 200 third-stage *H. polygyrus* larvae and i.v. administered 300 μg of anti-CD28 on days 0 and 3 and/or 440 μg and 200 μg of anti-CTLA-4 mAbs on days 0 and 3, respectively, or 100 μg of CTLA-4Ig on days 0 and 1. MLN tissues were stained at day 8 for $CD4^+$ T cells with GK1.5 and GC cells with the lectin, PNA. MLN PNA^+ GCs were quantified volumetrically as determined by the ratio of PNA^+ GC B cells to total lymphoid tissue at three planes per each mesenteric lymph node (MLN) tissues. These results were quantitated volumetrically for individual animals and the SE is shown for five mice per treatment group. GCs were quantitated individually and the mean and SE were determined for 5 mice per group. This experiment was repeated twice with similar results.


GERMINAL CENTER VOLUME (%)
0
10
20
30
40
50
WT CTRL
WT HP + CTRL Ab
WT HP + αCD28
WT HP + αCTLA-4
WT HP + αCD28 + αCTLA-4
WT HP + CTLA-4Ig

(days 0 and 3, respectively) and sacrificed on day 8 following *H. polygyrus* inoculation (Fig. 25). In addition, CTLA-4Ig administration was included with these treatment groups as a control for blocking both CD28 and CTLA-4 interactions following *H. polygyrus*-inoculation. Quantitative volumetric analyses were performed on individual MLN tissues (five mice per treatment group) and the mean GC volume was determined for each tissue. *H. polygyrus*-inoculated mice administered control Abs exhibited marked increases in GC formation, while *H. polygyrus*-inoculated mice administered either anti-CD28 Abs or anti-CTLA-4 mAbs showed greater than 50% reduction in GC formation (Fig. 25). The combined administration of both anti-CD28 and anti-CTLA-4 mAbs or CTLA-4Ig to *H. polygyrus*-inoculated mice blocked GC formation to untreated controls. In each of the treatment groups, the development of GCs correlated well with increases in $CD4^+$ T cell populations.

2. Administration of anti-CD28 or anti-CTLA-4 Abs reduces B cell activation leading to increased cell size and B cell surface MHC class II expression.

The reduction in GC formation at day 8 in *H. polygyrus*-inoculated mice administered either blocking CD28 or CTLA-4 mAbs indicated that these interactions might be critical for B cell activation. To determine whether B cell activation was affected by blocking CD28 and/or CTLA-4 interactions, MLN suspensions derived from *H. polygyrus*-inoculated mice given anti-CD28 and/or anti-CTLA-4 mAbs, control Abs or CTLA-4Ig were dual-stained with anti-MHC class II and B cell-specific anti-B220 (6B2) mAbs. MHC class II expression was increased in *H. polygyrus*-inoculated mice

administered control Abs, while mice given either anti-CD28 or anti-CTLA-4 mAbs exhibited comparably reduced levels of MHC class II expression (Fig. 26). The combined administration of both anti-CD28 and anti-CTLA-4 mAbs blocked elevations in MHC class II expression to untreated levels.

On day 8 after *H. polygyrus* inoculation, MLN cells were also dual-stained with FITC anti-CD4 and CY5-anti-IL-2R mAb. Increased expression of T cell IL-2R expression is an earlier indicator of T cell activation. To determine whether this indicator of T cell activation is regulated by CD28 and/or CTLA-4, the combination of anti-CD28 plus anti-CTLA-4 or anti-CD28 alone partially blocked elevations in surface IL-2R expression. In marked contrast, pronounced elevations in surface $CD4^+$ T cell IL-2R expression was detected when only anti-CTLA-4 mAbs were administered (Fig. 27).

3. Both CD28 and CTLA-4 interactions are required for *H. polygyrus*-induced elevations in T cell derived IL-4 production.

Inhibition of B cell MHC class II expression in *H. polygyrus*-inoculated mice following CD28 and/or CTLA-4 blockade suggested that T cell derived IL-4 production might also be inhibited, since increased B cell MHC class II expression is IL-4 dependent. To directly examine the effects of costimulatory molecule blockade during the *in vivo* type 2 immune response to *H. polygyrus*, MLNs from anti-CD28 and/or anti-CTLA-4 treated mice were collected on day 8 after *H. polygyrus*-inoculation and

Figure 26. Increases in B cell surface MHC class II expression are blocked following combined administration of anti-CD28 and anti-CTLA-4 mAbs as compared to control Abs. The mean fluorescence intensity as determined by FACS analysis is shown for each treatment group. MLN tissues were collected at day 8 after oral inoculation with 200 third-stage *H. polygyrus* larvae and MLN suspensions (five mice per treatment group) were pooled and dual-stained with FITC-anti-MHC class II and CY5-anti-B220.

Blocking CTLA-4 interactions inhibits IL 4-dependent increases in B cell MHCII expression

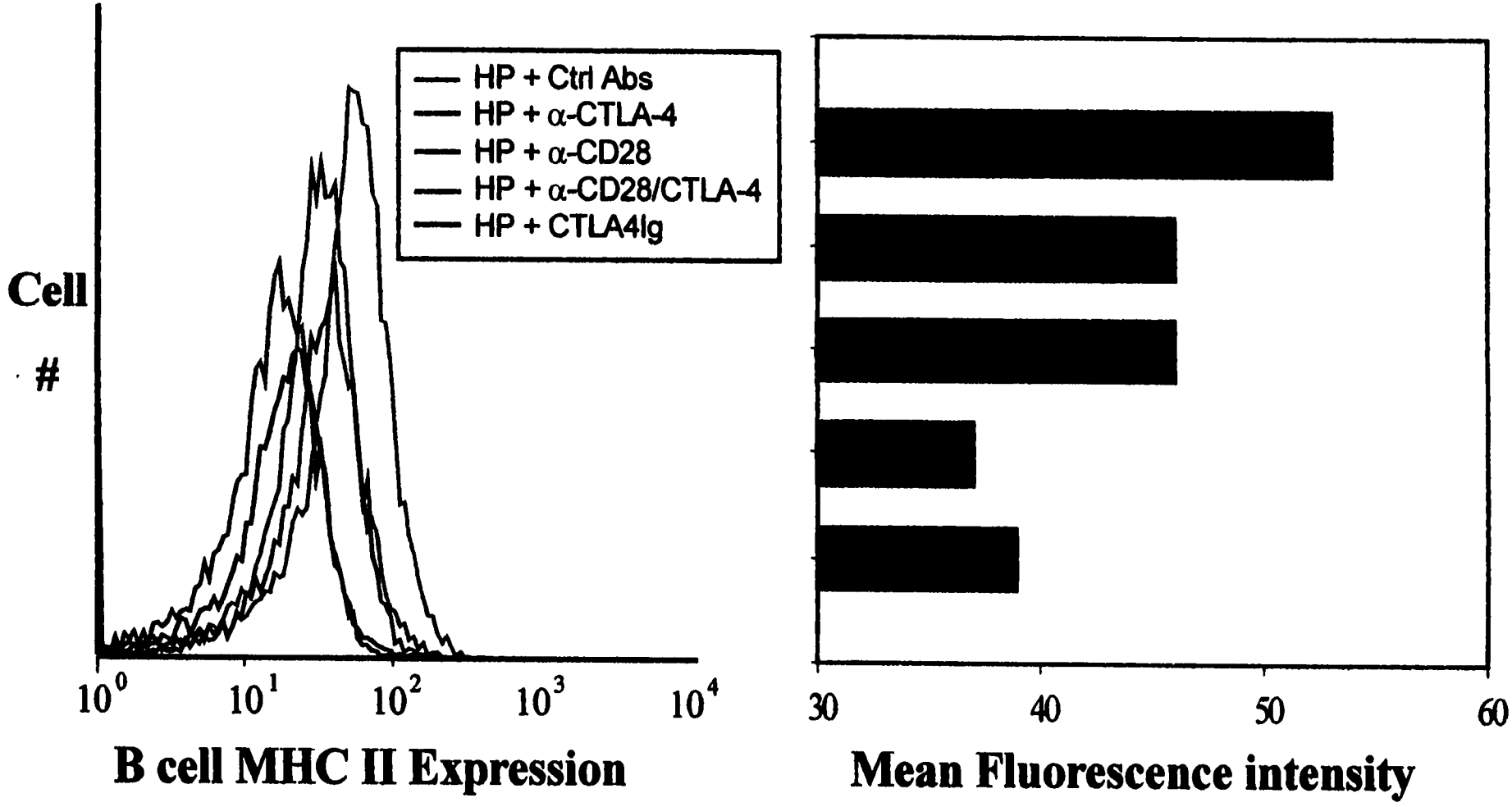
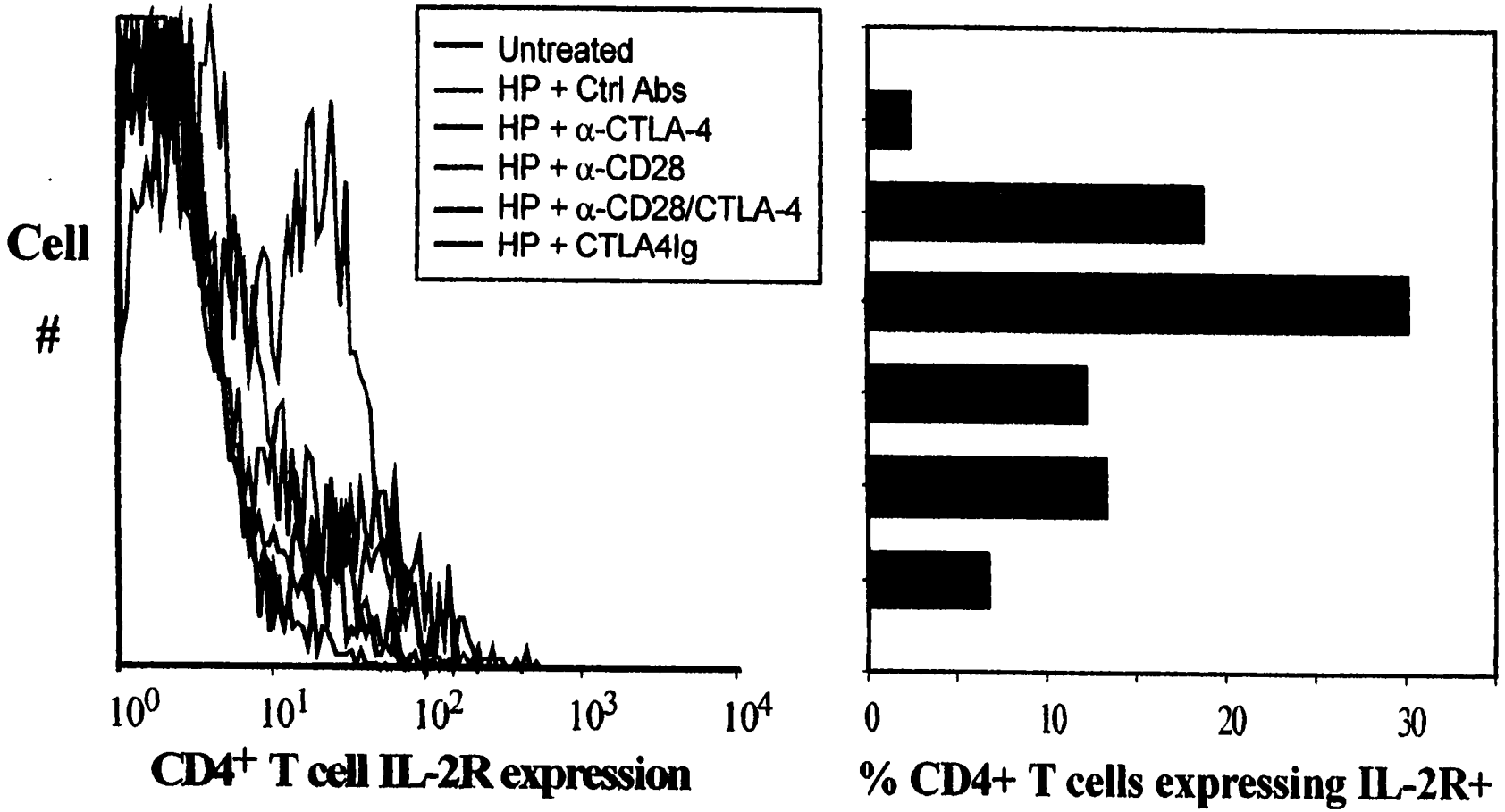


Figure 27. Marked increases in $CD4^+$ T cell surface IL-2R expression are observed in *H. polygyrus*-inoculated mice administered anti-CTLA-4 Abs as compared to inoculated controls. Combined administration i.v. of 300 μg of anti-CD28 on days 0 and 3, and 450 μg and 200 μg of anti-CTLA-4 mAbs on days 0 and 3, respectively, anti-CD28 Abs alone or 100 μg of CTLA-4Ig on days 0 and 1 inhibits T cell surface IL-2R expression as determined by FACS analysis. The percentage of $CD4^+$ T cells expressing IL-2R are shown. MLN tissues were collected at day 8 after *H. polygyrus*-inoculation and MLN suspensions (five mice per treatment group) were pooled and dual-stained for FITC-CD4 and biotinylated anti-IL-2R followed by strepavidin-PE.

Blocking CTLA-4 interactions markedly enhances CD4$^+$ T cell IL-2R expression after *H. polygyrus* inoculation



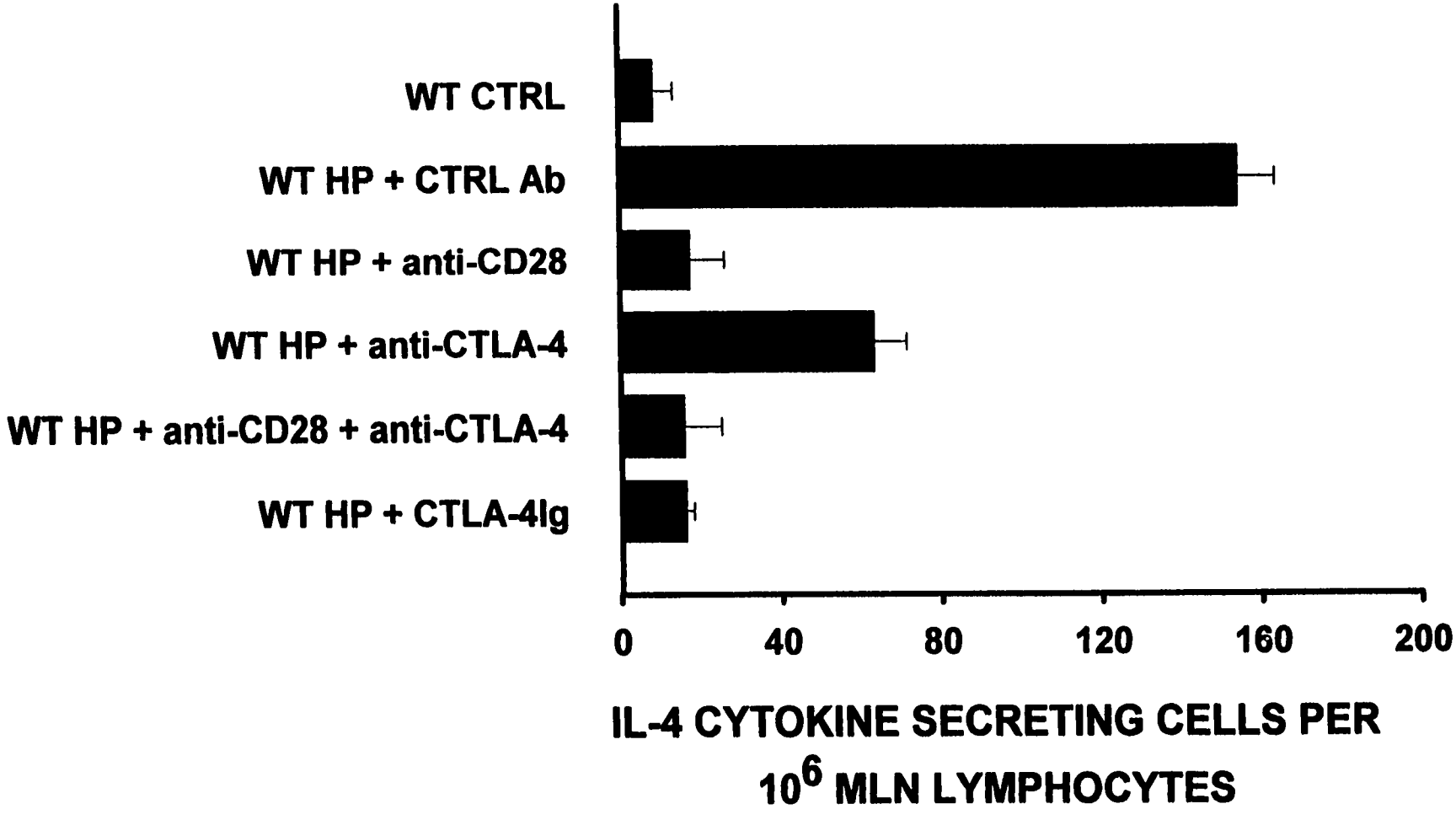
analyzed by ELISPOT for IL-4 protein secretion (Fig. 28). Administration of blocking CD28 mAbs or the combination of both anti-CD28 and anti-CTLA-4 mAbs to *H. polygyrus*-inoculated WT mice inhibited elevations in IL-4 secretion to untreated levels. Administration of blocking CTLA-4 mAbs to *H. polygyrus*-inoculated mice reduced IL-4 secretion by greater than 50% as compared to inoculated mice given control Abs. These studies suggest that both CD28 and CTLA-4 can promote T cell differentiation to IL-4 secretion in WT mice during an *in vivo* type 2 immune response to an infectious pathogen. Similar results were obtained in two independent experiments.

F. B7 ligand interactions are not required for memory T cell induction during the type 2 immune response to *H. polygyrus*

1. Administration of CTLA-4Ig during the primary immune response to *H. polygyrus* does not inhibit elevations in serum IgE or GC formation in the subsequent challenge response.

Our studies have demonstrated that B7 ligand interactions are required for the development of effector T cells from naïve T cells during the primary immune response. The *H. polygyrus* system is also an excellent model for studying memory T cell development, since the memory response is host protective, while the primary response is associated with chronic infection with *H. polygyrus*. Previous studies have shown that B7 costimulatory molecule blockade at the time of challenge immunization does not

Figure 28. The combination of anti-CD28 and anti-CTLA-4 Abs blocks elevations in IL-4 secretion on day 8 following *H. polygyrus* inoculation of WT mice. Intravenous administration of 300 μg of anti-CD28 Abs on days 0 and 3, blocks *H. polygyrus*-induced (200 third-stage larvae were orally inoculated) elevations in IL-4 secretion and similar administration of 450 μg and 200μg of anti-CTLA Abs on days 0 and 3, respectively, reduces IL-4 secretion. Tissues were collected on day 8 after inoculation and the number of IL-4 secreting cells per 10^6 MLN cells was determined by an ELISPOT assay without restimulation. The mean and SE are shown for five individual mice per treatment group.


WT CTRL
WT HP + CTRL Ab
WT HP + anti-CD28
WT HP + anti-CTLA-4
WT HP + anti-CD28 + anti-CTLA-4
WT HP + CTLA-4Ig
0
40
80
120
160
200
IL-4 CYTOKINE SECRETING CELLS PER
10^6 MLN LYMPHOCYTES

affect the subsequent host protective type 2 immune response (Gause et al., 1996). To examine whether B7 ligand interactions are required for memory T cell induction, *H. polygyrus*-inoculated mice were administered either CTLA-4Ig or control fusion protein at the time of primary immunization, treated with an anti-helminthic drug at day 13 (pyrantel pamoate), and challenged with *H. polygyrus* at day 40. Ten days later, mice were bled and killed, and tissues were collected and analyzed for the assessment of the Th2 response (see Table IV). As shown in Fig. 29, WT mice administered either CTLA-4Ig or control fusion protein exhibit comparable elevations in serum IgE levels and these increases are significantly greater than elevations following a single *H. polygyrus* inoculation.

To determine whether CTLA-4Ig administration during the primary immune response to *H. polygyrus*-inoculated mice influences the GC reaction in the subsequent challenge response, MLN tissues were collected at day 50. GC formation was comparable during the challenge response in *H. polygyrus*-inoculated mice administered CTLA-4Ig or control fusion protein (Fig. 30). These results show that blocking B7 ligand interactions during the primary response does not affect T cell development, B cell differentiation leading to IgE secretion or T cell dependent changes in lymphoid architecture leading to the GC reaction during the challenge immune response to *H. polygyrus*.

Table IV. Flow chart of experimental design to assess whether CTLA4Ig administration during the primary response influences the subsequent challenge host protective response.

	Day 0	Day 0,1	Day 13	Day 40	Day 40,41	Day 50
A	--	--	Rx [b]	--	--	Necropsy[c]
B	HP1[oa]	L6	↓	HP2°	--	↓
C	HP1°	CTLA4Ig		HP2°	--	
D	--	L6		HP1°	--	
E	--	CTLA4Ig		HP1°	--	
F	--	--		HP1°	L6	
G	--	--		HP1°	CTLA4Ig	

a. BALB/c mice (5/treatment group) were orally inoculated with 200 3rd stage larvae for the primary (1°) and/or challenge (2°) response and at the timepoints shown 100 μg of CTLA4Ig or L6 was administered i.v.

b. Mice in all treatment groups were administered pyrantel pamoate, an anti-helminthic drug, which effectively clears mice of all worms.

c. All mice were necropsied on day 50.

Figure 29. Blocking B7 ligand interactions during the primary *H. polygyrus* immune response does not block elevations in serum IgE levels during the host protective challenge immune response. *H. polygyrus*-inoculated WT mice (five per treatment group) were injected i.v. with 100 μg of CTLA-4Ig or the control fusion protein, L6, on days 0 and 1, treated with an anti-helminthic drug, and challenged by oral inoculation of 200 third-stage *H. polygyrus* larvae at day 40 following the protocol as shown Table IV. Serum IgE levels were measured by ELISA ten days after challenge immunization.

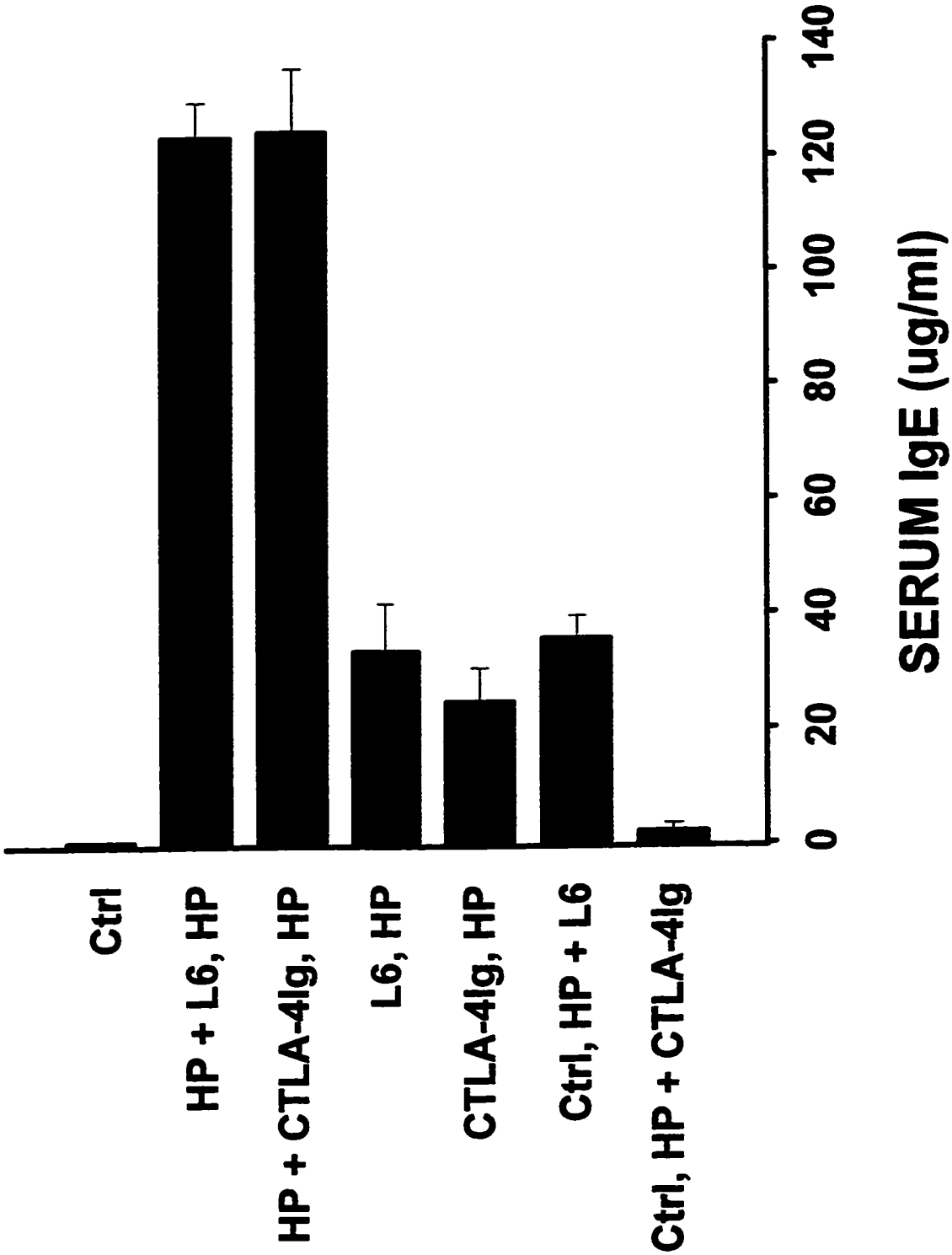
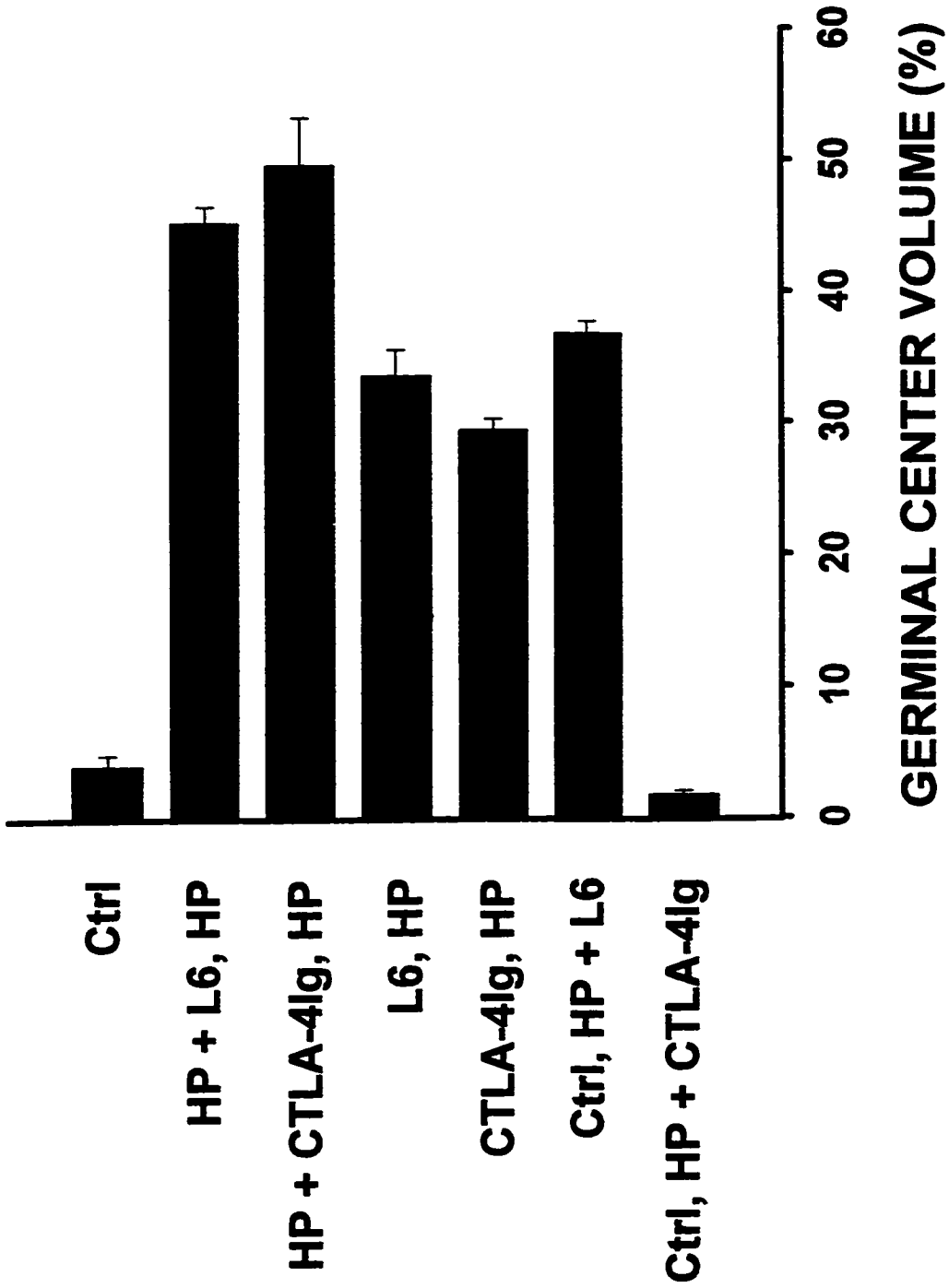

SERUM IgE (ug/ml)
0
20
40
60
80
100
120
140
Ctrl
HP + L6, HP
HP + CTLA-4Ig, HP
L6, HP
CTLA-4Ig, HP
Ctrl, HP + L6
Ctrl, HP + CTLA-4Ig

Figure 30. CTLA-4Ig administration during the primary immune response to *H. polygyrus* does not inhibit GC formation during the host protective challenge immune response. MLN tissues were collected at day 50 following challenge immunization as described in Fig. 29. Tissues were stained for $CD4^+$ T cells with GK1.5 and GC cells with the lectin, PNA. MLN PNA^+ GCs were quantified volumetrically as determined by the ratio of PNA^+ GC B cells to total lymphoid tissue at three planes per each mesenteric lymph node (MLN) tissues. GCs were quantitated individually and the mean and SE were determined for 5 mice per group. This experiment was repeated twice with similar results.


GERMINAL CENTER VOLUME (%)
0
10
20
30
40
50
60
Ctrl
HP + L6, HP
HP + CTLA-4Ig, HP
L6, HP
CTLA-4Ig, HP
Ctrl, HP + L6
Ctrl, HP + CTLA-4Ig

2. CTLA-4Ig administration during the primary immune response does not inhibit the development of the subsequent host protective immune response.

To determine whether a host protective challenge response develops following administration of CTLA-4Ig during the primary immune response, adult worm counts and egg production were analyzed at day 50. Although there was considerable variation in egg production during the primary response, some increase was observed in CTLA-4Ig treatment groups (Fig. 31). This is consistent with previous studies that anti-CD4 Ab treatment can slightly enhance egg production during the primary response with no increase in worm number (Urban et al., 1991). In the secondary response, egg production was almost completely inhibited in all treatment groups, including groups administered CTLA-4Ig during the primary response. Consistent with these findings, marked worm expulsion occurred in the challenged treatment groups, but no change in worm number was observed in the primary response in any of the treatment groups (Fig. 32). These findings suggest that B7 ligand interactions are not required for memory T cell induction during the type 2 immune response to *H. polygyrus*.

Figure 31. CTLA-4Ig administration during the primary immune response to *H. polygyrus* does not inhibit egg production during the host protective challenge immune response. CTLA-4Ig or the control fusion protein. L6, were administered in the primary response to *H. polygyrus*, mice were treated at day 13 with an anti-helminthic drug and subsequently challenged at day 40. Total eggs were enumerated from the contents of the cecum and large intestine ten days after challenge immunization. The data are expressed as the mean and SE of 5 mice per group.

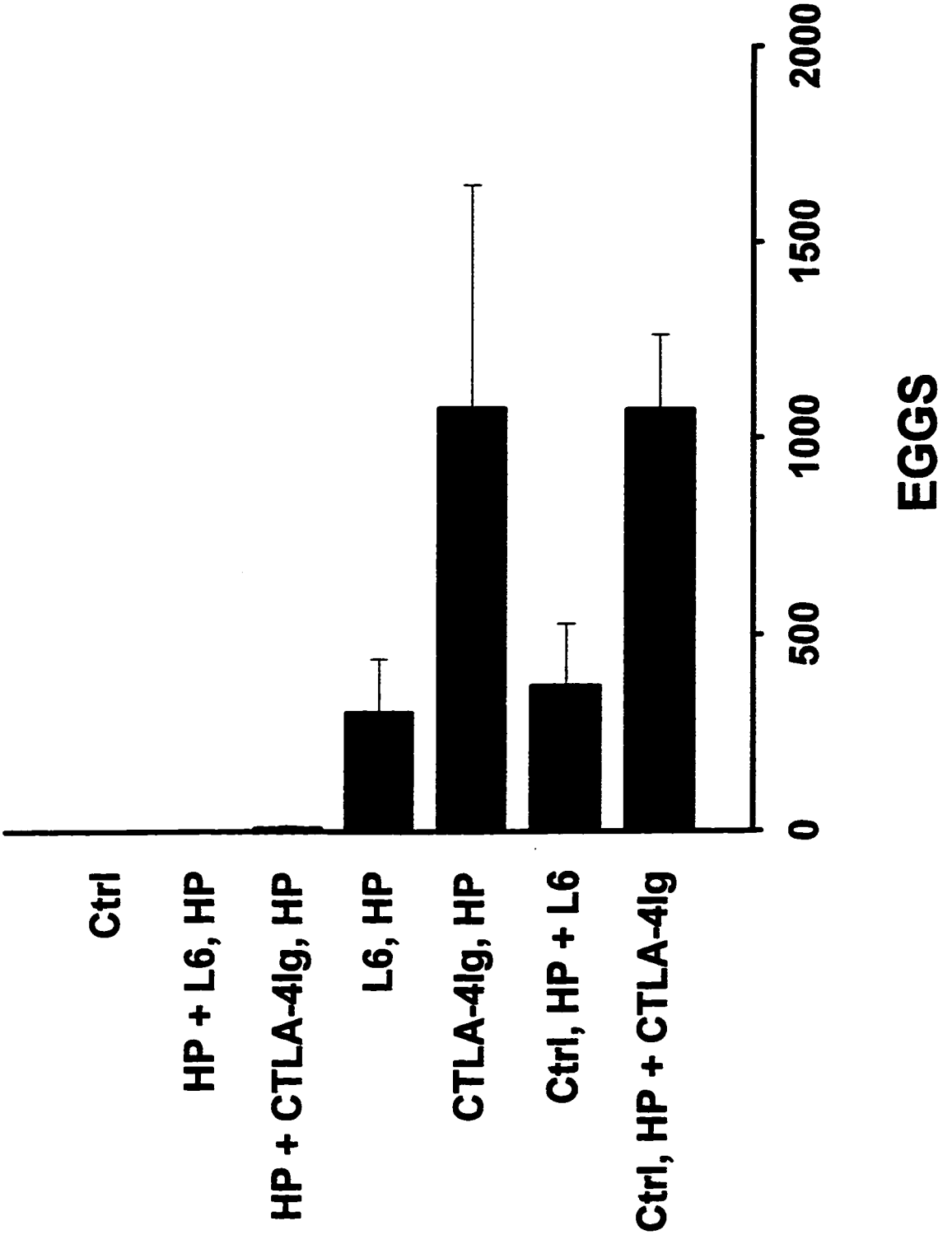
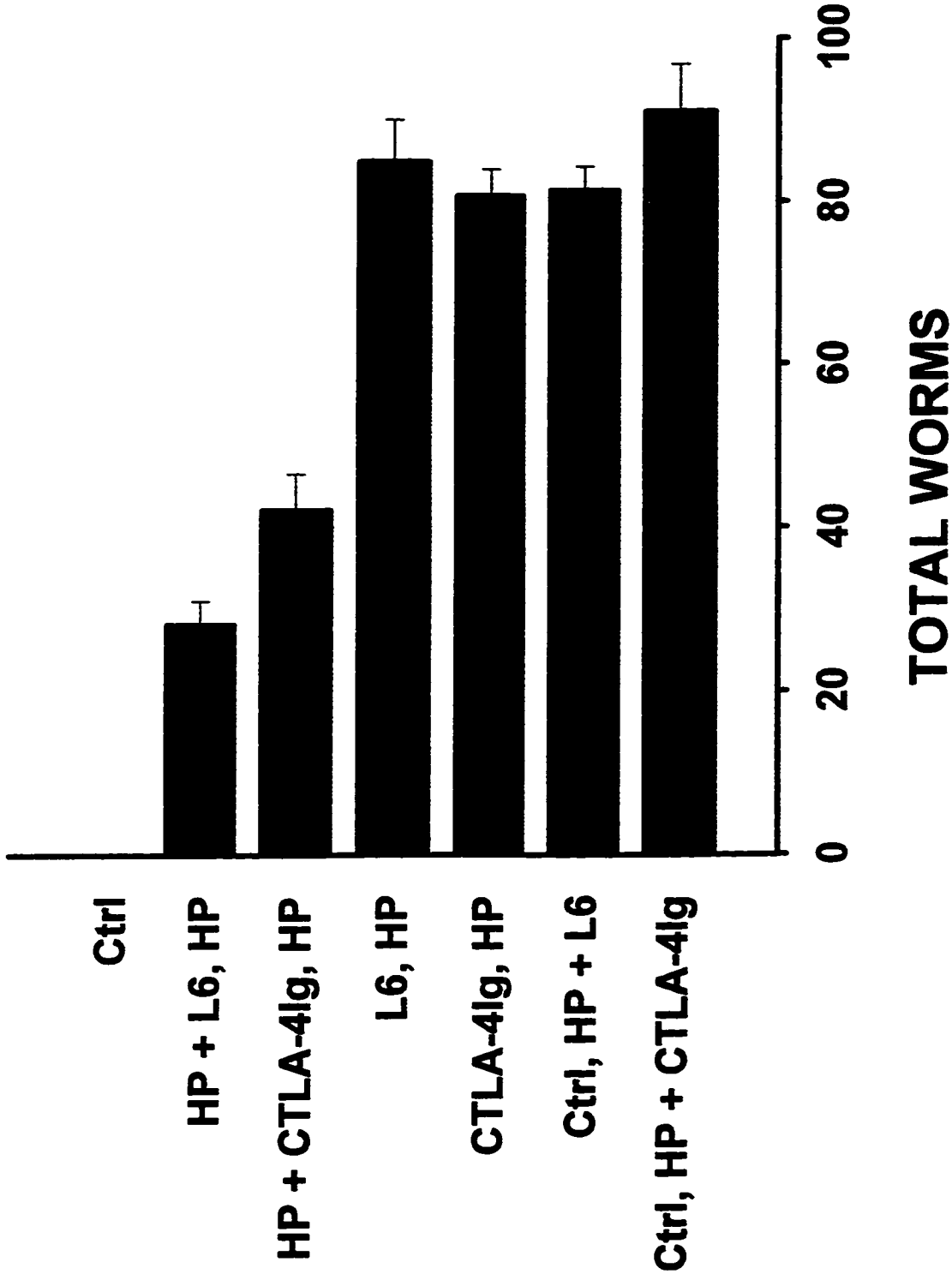

Ctrl
HP + L6, HP
HP + CTLA-4Ig, HP
L6, HP
CTLA-4Ig, HP
Ctrl, HP + L6
Ctrl, HP + CTLA-4Ig
0
500
1000
1500
2000
EGGS

Figure 32. Blocking B7 ligand interactions during the primary *H. polygyrus* immune response does not inhibit the development of adult worm expulsion in the host protective challenge immune response. 100 μg of CTLA-4Ig or the control fusion protein, L6, were i.v. administered on days 0 and 1 during the primary immune response to orally-inoculated 200 *H. polygyrus* larvae, given an anti-helminthic drug and challenged by oral inoculation with 200 *H. polygyrus* larvae day 40. Adult worms were collected 10 days after challenge and counted from the contents of the cecum and large intestine. The data are expressed as the mean and SE of 5 mice per treatment group.


Ctrl
HP + L6, HP
HP + CTLA-4Ig, HP
L6, HP
CTLA-4Ig, HP
Ctrl, HP + L6
Ctrl, HP + CTLA-4Ig
0
20
40
60
80
100
TOTAL WORMS

III. Discussion

This thesis has focused on the role of B7:CD28/CTLA-4 interactions in the development of the *in vivo* Th2 immune response to the nematode parasite, *H. polygyrus*. My findings provide insights into the function(s) of these molecules and suggest that each molecule acts differentially to influence T cell effector function. These results should provide a useful basis for the future development of immunotherapies targeting specific costimulatory molecules for the treatment of allergy and infectious diseases associated with the Th2 response. This discussion is divided into several sections. I will first interpret my results involving B7-1 versus B7-2 blockade, then progress to studies with CD28 versus CTLA-4 blockade, and finally address the role of costimulatory molecules in memory cell development.

A. Effects of blocking B7-1 and B7-2 interactions during a type 2 *in vivo* immune response

The results of our experiments show that the combination of anti-B7-1 and anti-B7-2 mAbs is sufficient to block the development of the type 2 *in vivo* primary immune response to the nematode parasite, *H. polygyrus*; however, blocking either ligand alone does not influence the course of the response. This vigorous immune response is localized in the enteric region and is characterized by marked elevations in serum IgG1 and IgE levels, blood eosinophils, intestinal MMC, and type 2 cytokines, including IL-4,

IL-5, and IL-9. The primary source of IL-4 is $CD4^+$ TCR $\alpha\beta$ cells, which secrete detectable quantities of this cytokine by day 8 after immunization (Svetic et al., 1993; Lu et al., 1996). IL-5 and IL-9 are derived from non-T cells, as well as T cells, and are first detected by 6 hours after oral inoculation (Svetic et al., 1993; Lu et al., 1996). Our studies also show that the combined administration of anti-B7-1 and anti-B7-2 mAbs at the early stages of *H. polygyrus* inoculation blocks the development of T and B cell responses, eosinophilia and mastocytosis.

Although the immune response to pathogens had not previously been examined with respect to individual B7 requirements, treatment of murine autoimmune disorders with anti-B7-1 and/or anti-B7-2 mAbs had yielded varied results. NOD mice treated with anti-B7-1 mAbs showed markedly accelerated diabetes, while anti-B7-2 mAb treatment blocked the development of this type 1 cytokine-associated disease (Matsumoto et al., 1994). In contrast, in an experimental EAE model, mice immunized with the proteolipid protein and treated with anti-B7-1 Ab exhibited skewing towards a type 2 response, while mice administered anti-B7-2 Ab showed increased disease severity associated with a type 1 immune response (Kuchroo et al., 1995). The differential effects of B7-1 versus B7-2 costimulation in these responses is in marked contrast to our findings that these two molecules can, to a large extent, substitute for one another during the immune response to a nematode parasite. One possible explanation for this difference is that these autoimmune responses are not strongly biased towards a type 1 or a type 2 immune response. Under such circumstances, B7-1 versus B7-2 signaling may be more likely to differentially affect T cell differentiation and the consequent immune response either by

qualitatively different effects of these molecules on T cell function or by preferential expression of B7-1 or B7-2 at a timepoint critical to disease development.

In contrast, the immune response to *H. polygyrus* is highly polarized. *H. polygyrus* is a natural murine parasite that rapidly elicits increased production of the type 2 cytokines, IL-5 and IL-9, by non-T cells. Within several days, T cells differentiate to produce IL-4 without an intermediate Th precursor or Th0 pattern (Svetic et al., 1993; Gause et al., 1995). The milieu produced by non-T cells at early stages of this response, including the cytokines they produce, apparently induces rapid T cell differentiation to IL-4 production in a manner analogous to the type 1 response that is triggered by macrophages producing IL-12 following their binding of conserved microbial structures, such as lipopolysaccharide (LPS) (Scott, 1993). In this strong immune response to *H. polygyrus*, where host:pathogen co-evolution has selected for a rapid and polarized host response, costimulation through either B7-1 or B7-2 can apparently elicit a similar and appropriate type 2 cytokine response. More generally, one can hypothesize that immune responses to pathogens associated with strong adjuvant-like effects may not be as easily influenced by such factors as B7-1 versus B7-2 costimulation.

The combined anti-B7-1 and anti-B7-2 mAb treatment of *H. polygyrus*-inoculated mice abolished GC formation in the mucosal lymphoid tissues on day 8, which is consistent with the inhibition of elevations in serum Igs, MHC class II expression on $B220^+$ cells, and B cell size. Recently, it was reported that early administration (days 1-3) of anti-B7-2 or anti-CD40L mAbs blocked GC formation in C57BL/6 mice immunized with the soluble protein Ag, NP-CG (Han et al., 1995). Whereas disrupting initial B7-2

ligand interactions with Th cells in the T-cell rich region of the cortex is sufficient to inhibit GC formation in this system, similar results are not observed in the *H. polygyrus* system, in which blocking both B7-1 and B7-2 is required to inhibit GC formation. In this strong type 2 immune response, B7-1 signaling can apparently substitute for B7-2 in its absence. Few studies have yet compared the expression of B7 molecules during the course of an *in vivo* immune response; however, B7-1 and B7-2 are expressed on a variety of cell types cultured *in vitro*, including activated B cells, dendritic cells, macrophages and T cells (Hathcock et al., 1994; Inaba et al., 1994). To unravel the distinct roles of B7-1 and B7-2 ligand interactions during different immune responses, the different expression patterns of these ligands on the cell surface of APCs, changes in their relative expression during the course of an immune response, and their signaling interactions with the costimulatory molecules CD28 and CTLA-4 should be further studied.

The ability to target specific CD28 ligands with mutant CTLA-4 fusion proteins may be useful in specifically modulating immune responses to develop novel immunotherapies. The recent identification of distinct CTLA-4 epitopes that bind either B7-1 or B7-2 (Linsley et al., 1994) provided the basis for the development of a novel mutant CTLA-4Ig construct, Y100F, which recognizes B7-1, but not B7-2. We have demonstrated the effectiveness of Y100F to substitute for anti-B7-1 Abs during the *in vivo* immune response to *H. polygyrus*, since the combined Ab treatment of *H. polygyrus*-inoculated mice with either anti-B7-1 and anti-B7-2 Abs or Y100F and anti-B7-2 Ab blocked T cell IL-4 secretion and B cell activation. The comparable effects of Y100F to

those of hamster Ig were due to specific blockade of B7-1 interactions and not other non-specific effects.

We have previously shown that blocking CTLA-4 ligand interactions by CTLA-4Ig administration inhibits T cell derived IL-4 production, B cell activation, and Ig secretion during the primary immune response to *H. polygyrus* (Lu et al., 1994). The current studies extend these findings by demonstrating that the inhibition of both B7-1 and B7-2 ligands yields similar results, which suggests that blocking both B7-1 and B7-2 interactions is sufficient to cause the immunosuppressive effects observed with CTLA-4Ig. Our studies also showed that other parameters of the type 2 immune response to *H. polygyrus*, including mucosal mastocytosis and increased blood eosinophils, were at least partly dependent on B7-1 or B7-2 interactions. It is possible, however, that other CD28/CTLA-4 ligands exist (Boussiotis et al., 1993), but in this particular response to a whole pathogen, B7-1 and B7-2 are apparently the primary ligands involved in mediating the type 2 immune response.

In summary, our results demonstrate that administration of the combination of anti-B7-1 and B7-2 mAbs can block the necessary costimulatory signals that trigger *in situ* $CD4^+$ T cell expansion and cytokine production, B cell activation leading to Ig secretion and GC formation, and associated increases in blood eosinophils and MMCs that are characteristic of a type 2 immune response. Our findings further suggest that neither B7-1 or B7-2 differentially influences the initiation of this *in vivo* immune response to a pathogen, and that in some cases, favoring B7-1 or B7-2 costimulation may not be useful in promoting a type 1 or a type 2 immune response.

B. Studies with B7-2KO mice

The results of our experiments with B7-2KO mice demonstrate differential B7-2 requirements for effector cell function during the Th2 mucosal immune response. B7-1 can support early T cell differentiation to cytokine production and also GC formation, such that *H. polygyrus*-inoculated B7-2 WT and B7-2KO mice exhibit similar responses during the first week of the immune response. However, increases in cytokine gene expression are inhibited almost to untreated control levels by day 14 after *H. polygyrus* inoculation of B7-2KO mice and serum IgE elevations are reduced. In contrast, at this late time point, GC formation and serum IgG1 levels are equivalent in *H. polygyrus*-inoculated B7-2KO and B7-2WT mice. The differences observed in serum IgE and IgG1 B7-2 dependency are increasingly pronounced at later stages of the response with serum IgE and IgG1 elevations differing by 30-fold and 2-fold respectively between *H. polygyrus*-inoculated B7-2KO and B7-2WT mice by day 24. These studies indicate that T cell-dependent effector cell functions vary in their B7-2 requirements during the same Th2 response to an infectious pathogen. Furthermore, these differences are increasingly pronounced at later stages of the immune response.

Although the immune response to infectious pathogens has not previously been examined in B7-2KO mice, recent studies with blocking anti-B7-1 and/or anti-B7-2 antibodies have generated varied results. Anti-B7-2 antibodies were found to block helminth-induced granuloma formation and Th2 cytokine expression following i.p. immunization with eggs from *S. mansoni* (King et al., 1995), while both anti-B7-1 and anti-B7-2 antibodies blocked the *H. polygyrus* response. One possible explanation is that

the stringency for B7-2 requirements is reduced during a mucosal response compared to a systemic response. Alternatively, the downregulatory effects of B7-2 deficiency may become more pronounced as the Th2 immune response progresses so that effector cell function becomes increasingly impaired at later stages of either the mucosal or systemic response. The prolonged *S. mansoni* immunization protocol involving sensitization and challenge required a later time point for analysis of cytokine gene expression and Th2 effector function than assessed in the *H. polygyrus* immune response. Our findings in the B7-2KO mice are consistent with this hypothesis since Th2 cytokine expression was markedly reduced at day 14 after *H. polygyrus* inoculation, although it remained elevated at levels near to those of B7-2WT mice at day 8 after *H. polygyrus* inoculation. Recent findings have also indicated that administration of anti-B7-2 antibodies for up to 4 weeks after immunization down-regulates the Th2 immune response *to L. major* in BALB/c mice (Ranger et al., 1996). Although serum Igs were not examined in this study, Th2 cytokines were markedly reduced, again consistent with our findings that late stages of the *H. polygyrus* immune response are B7-2 dependent.

H. polygyrus-inoculated B7-2 deficient mice showed marked reductions in serum IgE levels although serum IgG1 levels remained comparable to *H. polygyrus*-inoculated B7-2WT mice. These findings indicate differential regulation of B cell IgE and IgG1 secretion through B7 costimulatory molecule interactions. It has been proposed that B7 molecules may directly signal B cells (Fournier et al., 1997), although there is no direct evidence of B7 signaling and the lack of homology in B7 cytoplasmic domains between species suggests that this event is unlikely (Freeman et al., 1993b; June et al., 1993). It is

also possible that B7-2 interactions with CD28/CTLA-4 are required for T cell help leading to B cell IgE but not IgG1 secretion. Elevations in both serum IgE and IgG1 are $CD4^+$ T cell-dependent (Urban et al., 1991) and blocked by CTLA-4Ig administration (Lu et al., 1994) in the *H. polygyrus* immune response. However, serum IgG1 elevations are IL-4 independent, while increased serum IgE is IL-4 dependent (Finkelman et al., 1988; Finkelman et al., 1990), indicating that other unidentified T-dependent factors can mediate B cell differentiation to IgG1 secretion. Our findings suggest that this component(s) of T cell help is B7-2 independent.

The absence of B7-2 interactions also inhibited elevations in type 2 cytokine gene expression at day 14 but not day 8 after immunization. At both time points the cytokine response is primarily $CD4^+$ T cell dependent (Svetic et al., 1993) (Gause and Urban, unpublished), consistent with our findings that anti-B7-1 antibody blocked elevations in Th2 cytokine expression in *H. polygyrus*-inoculated B7-$2^{-/-}$ mice. Thus, at the initiation of the type 2 immune response, either B7-1 or B7-2 can provide signals for the development of cytokine-producing effector T cells. By day 14, the type 2 cytokine response has collapsed in *H. polygyrus*-inoculated B7-2 deficient mice. The complex interplay of interactions that can occur between CD28/CTLA-4 on T cells and B7-1/B7-2 on APCs during the *in vivo* immune response suggest a number of possible explanations for this observation. For example, it is possible that upregulated CTLA-4 on T cells may compete with CD28 providing a net negative T cell signal when only B7-1 is available. B7-1 and B7-2 bind different regions of CTLA-4 and B7-1 has a lower off rate than B7-2 (Van der Merwe et al., 1997; Linsley et al., 1994), suggesting they may affect CTLA-4

signaling differently. B7-1 transgenic mice have been shown to have a generalized immunosuppression which is consistent with the hypothesis that B7-1 signaling favors downregulation (Sethna et al., 1994). Alternatively, it is possible that expression of both cell surface B7-2 and B7-1 is required for sufficient total cell surface B7 expression to sustain the late response. We consider this latter possibility unlikely, since our previous studies have shown that B7 ligand interactions at the initiation of the response, and not at later stages, are important in promoting the Th2 response. In fact, CTLA-4Ig administration at days 4-5 after *H. polygyrus* administration does not inhibit the subsequent Th2 response and can enhance it (Lu et al., 1994; Gause et al., 1997b) (Gause and Urban, unpublished). Future studies will investigate whether CTLA-4 may provide a negative signal that down regulates Th2 cytokine expression at later stages of the immune response in *H. polygyrus*-inoculated B7-2KO mice.

In summary, our results demonstrate that, although B7-2 is not necessary for the initiation of a Th2 response, it is required for some effector cell functions during the later stages of the response. Specifically, elevations in T cell cytokines and B cell IgE secretion are blocked, while B cell IgG1 secretion and GC formation remain intact. These findings provide a possible explanation for some of the differences hitherto reported concerning the role of B7-2 *in vivo* and indicate that B7-2 can differentially influence effector cell function associated with a Th2 response. Future studies will be directed towards examination of the roles of CD28 and CTLA-4 in the downregulation of T cell effector function during the chronic type 2 immune response in B7-2 deficient mice.

C. CD28 dependence of T cell differentiation to IL-4 production varies with the particular type 2 immune response

The results of our experiments demonstrate that *in vivo* T cell differentiation to cytokine production during a type 2 immune response can be CD28 dependent or independent, depending on the specific Ag involved. Our findings show that T cell differentiation to IL-4 production and associated increases in serum Ig levels are blocked in CD28KO mice immunized i.v. with GaMδ. In contrast, CD28KO mice orally inoculated with *H. polygyrus* show marked increases in the cytokine gene expression and $TCR\alpha\beta^+$, $CD4^+$ T cell IL-4 protein secretion normally associated with this type 2 immune response. Furthermore, elevations in both serum IgG1 and serum IgE levels were comparable to those observed in the CD28WT controls.

The role of CD28 in the development of IL-4 producing T cells remains controversial. Previous studies demonstrated that CD28 signaling, in addition to TCR/CD3 signaling, is required to induce long term T cell clones and freshly purified T cells to produce IL-2 (Linsley et al., 1991; Harding et al., 1992; Reiser et al., 1992) and that signaling through the TCR in the absence of CD28 costimulation can induce anergy (Harding et al., 1992). In contrast, some murine IL-4 producing Th2 clones can produce IL-4 and proliferate in response to IL-4 in the absence of CD28 costimulation, apparently by using other costimulatory signals, including IL-1 (Kurt-Jones et al., 1987; Greenbaum et al., 1988; Weaver and Unanue, 1990; McArthur and Raulet, 1993). More recently, studies have suggested that CD28 activation promotes Th2 subset differentiation by

human $CD4^+$ T cells (King et al., 1995) and that CD28 signaling can drive T cells toward a Th2 phenotype even in the absence of IL-4 (Kalinski et al., 1995). Furthermore, T cells from CD28KO mice stimulated *in vitro* show reduced proliferation in response to anti-CD3 or allogeneic spleen cells (Green et al., 1994). Few studies, however, have yet examined T cell cytokine production *in vivo* in CD28KO mice.

Our findings suggest that the particular kind of type 2 immune response involved determines whether CD28 signaling is required for T cell differentiation to cytokine production and effector function. Although IL-4 producing T cells develop during both the immune response to GaMδ and the immune response to *H. polygyrus*, these two responses differ in important ways. The primary APC in the anti-IgD system is the B cell (Finkelman et al., 1990; Morris et al., 1994). Anti-IgD Abs cross-link mouse B cell membrane IgD, activating the B cells. It is internalized, processed and presented by the large number of B cells to goat IgG-specific naïve T cells, with the result that a wave of T cell activation is observed. T cell differentiation to IL-4 production rapidly occurs and can be readily detected by elevations in both gene expression and protein secretion by day 6 after immunization (Lu et al., 1995; Svetic et al., 1991). We have previously shown that this response is B7 dependent, since CTLA-4Ig administration at the initiation of the response blocks the development of IL-4 producing T cells. Because B cells are the principal APCs in this response, T cell differentiation may be particularly dependent on the CD28 costimulatory molecule. It has been suggested that activated B cells do not function as optimal APCs and in some cases may even induce tolerance (Matzinger, 1994), perhaps because they are lacking certain signaling molecules that promote T cell

cytokine production. Other APCs, such as dendritic cells or macrophages, may be able to provide additional signals or combinations of signals, lacking in B cells, that can function as costimulatory signals, substituting for CD28.

As in the anti-IgD system, CTLA-4Ig administration blocks T cell differentiation to IL-4 production during the immune response to *H. polygyrus* (Lu et al., 1994). However, although the primary APCs involved in the *H. polygyrus* immune response have not yet been identified, other cell types, besides B cells, are almost certainly involved, including dendritic cells and macrophages. Also, a non-B/non-T cell cytokine response associated with elevations in IL-3, IL-5 and IL-9 is detected within 6 hours after *H. polygyrus* (Svetic et al., 1993). As early as 4 days after inoculation, elevations in IL-4 gene expression which is T cell derived, are detected (Svetic et al., 1993). The presence of additional APCs and the milieu associated with activation of other non-T cells may provide the signals (not present in the anti-IgD immune response) required to substitute for the absence of CD28 signaling. Although TCR signal strength has also been proposed as an important factor in Th cell differentiation (Lenschow et al., 1996), since both *H. polygyrus* and anti-IgD induce pronounced T cell responses, it is unlikely that the extent of TCR ligation influences CD28 dependence in these two systems.

It is also possible that the site of immunization, mucosal versus systemic, may be important. Certain APCs in the enteric region may be able to provide costimulatory signals to T cells in the absence of CD28. Although some evidence has suggested that the mucosal immune response is qualitatively different from the systemic immune response (Xu-Amano et al., 1992; Fujihashi et al., 1993), most of our studies have suggested that

the two immune responses are similar (Finkelman et al., 1990; Gause et al., 1995). Very recently, studies of the systemic immune response to *L. major* also demonstrated the development of IL-4 producing cells in BALB/c CD28KO mice (Brown et al., 1996), suggesting that in this very different immune response to a pathogen, T cells are again able to differentiate to IL-4 production in the absence of CD28. We have also examined the immune response to *H. polygyrus* in BALB/c CD28KO mice, and we observed pronounced T cell IL-4 elevations and increases in serum IgE and IgG1 levels (Gause, unpublished observations). In another study, CD28 deficient transgenic T cells could initiate, but not sustain, primary proliferative responses *in vitro* (Lucas et al., 1995). Our *in vivo* studies of the kinetics of serum Ig levels in CD28KO and WT mice suggests that the response is comparably sustained at later time points of the immune response to *H. polygyrus*, indicating that additional signals may be available *in vivo* that function to maintain the strong immune response.

Since B7-dependent type 2 immune responses to *H. polygyrus* and *L. major* can develop in CD28KO mice, it will be important in both systems to determine whether another B7 ligand is providing the costimulatory signal. CTLA-4 is certainly a candidate, despite its identification as a negative signal under some conditions (Tivol et al., 1995; Waterhouse et al., 1995). Given that a number of cell surface molecules, including Fas and CD40, can provide either positive or negative signals (Lynch et al., 1995; Schattner et al., 1996; Hess and Engelmann, 1996), CTLA-4 may be capable of providing positive signals during the strong immune responses characteristic of pathogens. Alternatively, compensatory mechanisms may have developed in CD28KO mice that permit the

development of IL-4 producing T cells during the immune response to *H. polygyrus*, but not GaMδ. Future studies will be required to directly address these questions.

D. Studies with CTLA-4Ig in CD28KO mice

The B7 ligands CD28 and CTLA-4 are expressed primarily on T cells and show only 21-31% homology within the same species, although rigid conservation is observed within a 16 amino acid stretch of the CDR3-like domain that includes the MYPPPY motif. CD28 is constitutively expressed on T cells, whereas CTLA-4 is induced following T cell activation. T cells isolated from CD28-deficient mice exhibit impaired responses to alloantigen, nominal antigen, and anti-CD3 Abs (Green et al., 1994). However, GC formation is blocked in CD28KO mice in response to immunization with the hapten-carrier protein nitrophenyl-acetyl (NP)-chicken γ globulin (Ferguson et al., 1996).

Although these reports suggest major defects in the immune system of CD28KO mice, other studies involving infectious pathogens suggest a more intact immune response (Shahinian et al., 1993). Following *H. polygyrus* inoculation, T cell derived IL-4 production and serum IgG1 and IgE levels are comparable in CD28KO and CD28WT mice. GC formation due to *H. polygyrus*-inoculation was detected in CD28-deficient mice, although it was reduced as compared with CD28WT controls. Similarly, infection of BALB/c CD28KO mice with *L. major* induces T cell IL-4 secretion as measured by the ELISPOT assay. Interestingly, in this same system, T cell IL-4 production was not detected during *in vitro* restimulation experiments suggesting that factors present *in vivo*,

but not *in vitro*, were required to maintained the CD28-independent response (Brown et al., 1996). Taken together, these results suggest that, in strong immune responses to infectious agents, other factors are present that can substitute for the absence of CD28 costimulation during T cell activation, and that can lead to IL-4 production and the development of a type 2 immune response. The finding that T cells from CD28KO mice can produce IL-4 is intriguing. To examine whether B7-dependent costimulation occurs in the absence of CD28, *H. polygyrus*-inoculated mice were administered CTLA-4Ig and the resulting type 2 immune response was examined. Our results have been inconsistent and further studies are necessary to resolve this paradox.

The possibility exists that CTLA-4 could provide the positive initial costimulatory signal in the absence of CD28. Recent *in vitro* studies have reported that B7-1 can drive clonal expansion of CD28-deficient T cells, which can be blocked with anti-CTLA-4 antibody, suggesting that CTLA-4 can, under some circumstances, provide a positive costimulatory signal in CD28-deficient mice (Wu et al., 1997; Linsley et al., 1992).

Our studies suggest that, in CD28KO mice, CTLA-4 may provide the necessary costimulatory signaling required for the development of IL-4 producing T cells during the primary immune response to *H. polygyrus*. However, CTLA-4 deficient mice exhibit pronounced lymphoproliferation and lymphoid infiltration, as well as associated tissue destruction of multiple organs. This pathology suggests that CTLA-4 may be required for negative signaling (Waterhouse et al., 1995; Tivol et al., 1995). Furthermore, crosslinking of CTLA-4 on resting T cells can inhibit IL-2 production and cell proliferation induced by anti-CD3 and anti-CD28 Abs (Krummel and Allison, 1996).

One possibility is that CTLA-4 may give either a positive or negative signal, depending on the presence or absence of additional signals. This concept would be similar to positive and negative signaling effects that have been demonstrated with Fas (Lynch et al., 1995) and CD40/CD40L (Hess and Engelmann, 1996; Schattner et al., 1996). Alternatively, CTLA-4 may act as a partial agonist, delivering an attenuated signal compared to that delivered by CD28. If this partial agonism were the case, then the upregulation of CTLA-4 with its higher affinity for B7 molecules, might competitively inhibit binding of B7 to CD28. It is also possible that CD28 and CTLA-4 function differently in the absence of their homolog, or when absent throughout ontogeny as opposed to when blocked acutely during adulthood. For instance, CTLA-4 could be required during T cell development for optimal deletion of autoreactive T cells in the thymus or the periphery; however this requirement is unlikely, given recent findings which suggests that thymocyte development is normal in CTLA-4KO mice (Chambers et al., 1997). Whatever the mechanism of CTLA-4-mediated downregulation of lymphocyte activation, the marked lymphoproliferation observed in CTLA-4KO mice is probably due to CD28-mediated activation of peripheral T cells, particularly given the recent findings that CTLA-4Ig administration to CTLA-4KO mice blocked the development of lymphoproliferative disorders (Tivol et al., 1997).

The induction of CTLA-4 after the initiation of an immune response suggests that it plays an important function during the progression of the response. Stronger TCR interactions, caused by higher TCR avidity or occupancy (Linsley et al., 1996), and CD28 signaling (Lindsten et al., 1993) can upregulate CTLA-4 expression, which suggests that

both elevated antigen concentrations and costimulatory signaling are associated with increased CTLA-4 engagement. If CTLA-4 provides a negative signal in adult animals, then it may dampen an otherwise tissue-damaging response (this would also hold true if CTLA-4 acts as a partial agonist). As discussed earlier, since B7-1 probably has a more productive interaction with CTLA-4 than CD28, it may be particularly important in mediating this downregulatory effect at later stages of the immune response. Increased cell-surface expression of B7-1 would then attenuate an immune response, as observed in transgenic mice overexpressing B7-1 (Sethna et al., 1994).

E. CD28/CTLA4 blockade in CD28WT mice

Previous studies in our laboratory demonstrated that, in CD28KO mice, the type 2 systemic immune response to anti-IgD is blocked, while the type 2 mucosal immune response to *H. polygyrus* remains intact (Gause et al., 1997a). These studies suggested that a B7 ligand other than CD28 provided the necessary costimulatory signals required for T cell differentiation to effector function. The likely candidate to provide this positive signal is CTLA-4. Given our conflicting results in the CD28KO mice administered CTLA-4Ig and the availability of blocking anti-CD28 and anti-CTLA-4 mAbs with recently published *in vivo* inhibitory effects (Perez et al., 1997; Leach et al., 1996; Perrin et al., 1996), the individual roles of CD28 and CTLA-4 were examined with antibody intervention experiments. In these studies, *H. polygyrus*-inoculated BALB/c mice were administered blocking anti-CD28 and/or anti-CTLA-4 mAbs. Our results show that administration of anti-CD28 mAbs to *H. polygyrus*-inoculated mice was sufficient to

block elevations in T cell IL-2R expression and IL-4 production, and B cell MHC class II expression and GC formation. Similar administration of anti-CTLA-4 mAbs to *H. polygyrus*-inoculated mice markedly increased T cell IL-2R expression; however, T cell differentiation to effector function was partially inhibited.

While few studies have examined the individual roles of CD28 and CTLA-4 during the immune response to pathogens, treatment of tumor and autoimmune disease models with anti-CTLA-4 Abs has produced evidence to suggest a downregulatory role for CTLA-4. Administration of anti-CTLA-4 mAbs (clone 9H10) to mice with well-established tumors resulted in tumor rejection (Leach et al., 1996), which suggests that blockade of CTLA-4 enhances the anti-tumor immune response by inhibition of its downregulation function. Similarly, in a model of experimental allergic encephalomyelitis, administration of anti-CTLA-4 Abs exacerbated autoimmunity and promoted encephalitogenic cytokine production (Perrin et al., 1996). While the clinical disease outcomes varied in the tumor and autoimmune disease models, these studies demonstrated that CTLA-4 has an important role in downregulating the immune response. The functional role of CTLA-4 was further illustrated with the development of CTLA-4KO mice which exhibit pronounced lymphoproliferative disorders and die within 3-4weeks (Tivol et al., 1995). The strictly downregulatory role in these types of responses is in marked contrast to our findings and those of others (Wu et al., 1997) that CTLA-4 may provide a costimulatory signal in CD28KO mice at the initiation of the immune response to *H. polygyrus*. In marked contrast, blockade of CTLA-4 in *H. polygyrus*-inoculated CD28WT mice, consistently caused marked increases in T cell

surface IL-2R expression and also increased IFN-γ and decreased IL-4, perhaps not surprising since increased IL-2 signaling is often associated with the type 1 response (Mossmann and Coffman, 1989). Increased IL-2 signaling is also associated with T cell apoptosis (Refaeli et al., 1998). It may be that CTLA-4 dampens the T cell response sufficiently to prevent T cell hyperresponsiveness leading to a deviated T cell differentiation state. Future studies should investigate whether the diminished IL-4 secretion is a result of immune deviation or T cell depletion resulting from IL-2 mediated programmed cell death.

Although our studies demonstrate the development of a type 2 immune response in *H. polygyrus*-inoculated CD28KO mice, our results in the wild-type mice show that CD28 is required for T cell differentiation in genetically intact mice in which the immune system has developed in the presence of CD28. Administration of anti-CD28 mAbs to *H. polygyrus*-inoculated WT mice is sufficient to block T cell IL-2R expression and IL-4 production as well as reduce MHC class II expression and GC formation, which implies that B7-CD28 interactions are critical for the initiation of the type 2 immune response to *H. polygyrus*. There are several possible explanations for the discrepancy between the results in the CD28KO versus CD28WT mice treated with anti-CD28 Abs including the possibility that other cell surface molecules, in particular CTLA-4 and HSA, may be able to support Th effector cell development in CD28KO mice.

In summary, the results of our studies in *H. polygyrus*-inoculated wild-type mice show that CD28 is required for the development of the primary type 2 immune response. CTLA-4 is also required for the maintenance of the primary response with the anti-

CTLA-4 treatment associated with T cell hyperresponsiveness, but loss of effector function.

F. The role of B7 ligand interactions in memory T helper cell development

The *H. polygyrus* system is a particularly useful model for studying the memory response, since the T-dependent memory response is host protective, while the primary response is associated with a chronic infection (Urban et al., 1992). Previous studies in our laboratory have shown that B7 ligand interactions are required for the development of effector T cells from naïve cells (Lu et al., 1994; Greenwald et al., 1997) at the initiation of primary immune response to *H. polygyrus* (Lu et al., 1995). Further studies have demonstrated that blocking B7 ligand interactions at the time of the challenge response does not effect the memory response that is associated with worm expulsion (Urban et al., 1992). In this project, our studies have investigated whether B7 ligand interactions are required for the induction of memory T cells from naïve cells, during the primary immune response to *H. polygyrus*. Memory T cell induction and the associated host protective response remains intact following administration of CTLA-4Ig during the primary immune response to *H. polygyrus*.

Recent studies of mice deficient for heat-stable antigen (HSA) and/or CD28 suggest that B7 ligand interactions are required for the development of effector cytotoxic T cells, while either HSA or B7 ligand interactions can provide the required costimulatory signaling for memory cytotoxic T cell induction (Wu et al., 1998). It is suggested that a strong stimulus provided by B7 is required for effector T cell

development, while a weaker costimulation signal which can be provided by HSA, can be utilized for the induction of memory T cells (Liu et al., 1997). Our findings are consistent with this hypothesis, and we are currently investigating whether HSA plays a role in memory helper T cell induction in the *H. polygyrus* system.

Although our results demonstrate that B7 ligand interactions are not required for memory T cell induction during an immune response to an infectious pathogen, the recent finding by Keane-Myers et al. (1997) suggests that a memory type 2 response may instead be blocked by CTLA-4Ig administration in a T-dependent murine asthma model. In this Th2 response, CTLA-4Ig administration at the time of primary systemic immunization blocked the allergic response to OVA aspirant challenge administered 14 days later.

The processes that lead to B7-dependent memory cell development in murine asthma and B7-independent memory cell development in *H. polygyrus* are uncertain. One possibility is that the route of immunization, involving first a systemic and second a mucosal immunization, in the allergic model may cause naïve T cells in the lung to be particularly important in triggering the challenge response. This is unlikely, however, since CTLA-4Ig also blocks the allergic response when administered at the initiation of a second aspirant challenge (M. Wills-Karp and W. C. Gause, unpublished observations). Alternatively, it could result from the differences in the Ag involved. *H. polygyrus* is a live parasite, and although only T cells have been demonstrated to produce IL-4 in this system, a strong cytokine response by non-T cells, including elevations in IL-5, IL-6, and IL-9, is rapidly observed following infection (Svetic et al., 1993). These adjuvant-like

properties of *H. polygyrus* could circumvent a B7 requirement that would be sustained in a challenge mucosal immune response to a peptide such as OVA. Future studies will be necessary to determine whether differential B7 requirements are due to adjuvant-like factors associated with *H. polygyrus* but not peptide antigens.

The "strength of signal" hypothesis posits that increased signaling through the TCR and/or costimulatory molecules is associated with a transition from a Th1 to a Th2 response (Roth et al., 1993). However, the small OVA dose (10μg) required to induce a Th2 allergic response in the murine asthma model just discussed (Keane-Myers et al., 1997) and the recent findings that both Th1 and Th2 cytokine production are inhibited when B7 ligand interactions are blocked in either the strong immunogenic response to anti-IgD Abs (Lu et al., 1995) or the nematode parasite, *H. polygyrus* (Gause et al., 1995), suggests that other mechanisms are also involved. In particular, T cell activation blockade, rather than immune deviation, following CTLA-4Ig administration during the primary Th2 response (Greenwald et al., 1997; Lu et al., 1995; Keane-Myers et al., 1997; Gause et al., 1995) is more consistent with the hypothesis that under some circumstances CD28 provides a signal required for *in vivo* T cell activation leading to effector function that cannot be replaced by strong TCR signaling. Other factors may be important, such as the genetic background, which in some immune responses may influence the T cell cytokine pattern. Although B7 ligand interactions may often be required for T effector cell development from naïve cells during primary *in vivo* responses, strong T cell signaling during a memory response may be sufficient for the response to proceed to the T effector cell stage in the absence of B7 signaling. Correspondingly, when immune

responses where strong T cell signals contributed by adjuvant-like factors are absent, such as certain allergic or autoimmune disorders, memory T cells may be more B7 dependent.

In summary, recent findings suggest that blocking B7 ligand costimulatory signals may be effective in downregulating an ongoing or a memory immune response. The conditions that contribute to B7 dependence are uncertain, but they probably involve: 1) the type of antigen inducing the response, 2) the lymphoid microenvironment; and/or 3) the T cell cytokine pattern elicited during the response. The capability to block T cell effector function during an ongoing or memory response for the development of immunotherapies is significant, since many diseases are not identified until after they are well-developed. It will important in future studies to further define the mechanisms by which some immune responses remain B7 dependent and others do not.

G. Summary

In this project, we examined the functional roles of the costimulatory molecules, B7-1 and B7-2 on APCs and CD28 and CTLA-4 on T helper cells, during the primary and memory immune response to *H. polygyrus*. Following primary inoculation with the nematode parasite *H. polygyrus*, the host mounts a vigorous type 2 immune response characterized by intestinal mastocytosis, blood eosinophilia, elevations in T cell IL-2R expression, *in situ* $CD4^+$ T cell expansion and IL-4 production, and increases in B cell MHC class II expression, MLN GC formation and serum IgE and IgG1 levels.

The role of the B7 ligands was initially examined via antibody intervention studies and with knockout experiments. Following *H. polygyrus* inoculation, the results

demonstrate that either B7-1 or B7-2 can provide the necessary costimulatory ligand interactions required for elevations in serum IgE and IgG1 levels, MLN GC formation, in situ $CD4^+$ T cell expansion, blood eosinophils, intestinal mast cells and T cell MLN and PP IL-4 gene expression and protein secretion. However, blocking both B7-1 and B7-2 interactions, with either blocking antibodies or knockout mutations, inhibits elevations in this type 2 immune response.

To complement the antibody intervention studies, our results in the B7-2 deficient mice confirm that B7-2 is not is required at the initiation of the immune response to *H. polygyrus*; however, B7-2 has a role in effector cell functions later in the immune response. In particular, elevations in T cell cytokines and B cell IgE secretion are blocked, while B cell IgG1 secretion and GC formation remain intact. These findings lend insight and provide a possible explanation for the variability in B7-2 dependence reported with different antigens.

To further define the role of costimulatory molecules during *in vivo* immune responses, the functional roles of the T cell costimulatory molecules, CD28 and CTLA-4, were also examined. In CD28KO mice, T cell differentiation to IL-4 production and associated increases in serum IgE and IgG1 levels were intact during the mucosal immune response to *H. polygyrus*, but inhibited following systemic immunization with goat anti-IgD Abs. These results indicate that the specific kind of type 2 immune response determines whether T cell differentiation to IL-4 production can occur in CD28KO mice. In our studies in *H. polygyrus*-inoculated BALB/c CD28KO mice, marked increases in T cell IL-4 production and elevations in serum IgE and IgG1 were observed; however,

conflicting results were obtained following administration of the fusion protein CTLA-4Ig to *H. polygyrus*-inoculated CD28KO mice. To clarify this issue, Ab intervention studies with blocking CD28 and/or CTLA-4 Abs were administered to *H. polygyrus*-inoculated wild-type animals. Our results demonstrate that CD28 is required for T cell differentiation to effector function during the type 2 immune response to *H. polygyrus* and that CTLA-4 also has a different and required role for an optimal response.

The final section of this project addresses the role of costimulatory molecules in the memory T cell development. Our results demonstrate that blocking B7 ligand interactions with CTLA-4Ig during the primary response does not influence the development of the subsequent challenge response as detected by adult worm counts, egg production, GC formation and serum IgE levels. These results have important implications for the development of therapeutic interventions, since blocking B7 ligand interactions in certain responses may only transiently inhibit the immune response. It is possible that other costimulatory molecules such as HSA may play a role in the memory T cell development in the *H. polygyrus* immune response or that costimulatory molecules do not provide critical signals for memory T cell induction.

A clearer understanding of how costimulatory molecules influence the production of IL-4 by T cells may provide important insights into the development of immunotherapies that can regulate type 2 responses. Future studies should include: 1) determination of whether distinct effects of blocking B7-1 versus B7-2 result from differences in the expression of these molecules or from differences in the way that they signal T cells; 2) elucidation of the circumstances and mechanisms by which CTLA-4

influences T effector cell function during the *in vivo* immune response; 3) analysis of the differences in CD28 and CTLA-4 signaling pathways; and 4) characterization of the critical T cell costimulatory molecule interactions required for memory T cell development.

H. Conclusions

The focus of my dissertation is to determine the individual functional roles of the costimulatory B7: CD28/CTLA-4 pathway in T cell activation leading to IL-4 production during the *in vivo* type 2 mucosal immune response to the nematode parasite, *H. polygyrus*. Until recently few studies had examined directly the specific cellular interactions required for T cell activation during immune responses to infectious pathogens. Our understanding of *in vivo* T cell activation has relied primarily on studies involving immunization with protein antigens such as OVA or chicken γ-globulin (Refaeli et al., 1998; Han et al., 1995). While these types of studies provide insight into the cellular interactions required for T cell activation *in vivo*, the immune response to a live pathogen may be more complex. Several groups recently have examined requirements for T cell activation in other parasite model systems, most notably, *S. mansoni* and *L. major* (Subramanian, et al., 1997; Brown et al., 1996). In both of these models, however, the parasite naturally infects humans and had to be adapted to infect murine species. It is possible that alterations of the parasite have occurred during the adaptation to infect murine species. Furthermore, the murine host response may not reflect the response of the natural host. Our studies in the *H. polygyrus* system have

attempted to avoid the concerns of using a non-infectious protein antigen or an infectious parasite from another host species by examining the cellular interactions required for *in vivo* T cell activation during the immune response to a natural murine parasite.

While *H. polygyrus* is strictly a murine pathogen, it is also considered a useful model for human nematode infections, particularly hookworm infections. Two species of hookworms cause disease in humans, *Necator americanus* and *Ancylostoma duodenale* (Walsh, 1984). These hookworms infect nearly one fourth of the world's population with an estimated prevalence of 700,000 to 900,000 cases per year and between 50,000 and 60,000 deaths (Maxcy-Rosenau-Last, 1992). Given the complexity of the host-parasite interactions, vaccine development based on cloning protective antigens, in which the goal is to promote natural immune responses, has lead to few successes. Currently, the research focus for vaccine development against many parasitic diseases has shifted towards manipulating the local cytokine environment that fosters initial development of the immune response. Depending on the particular type of parasite or stage of parasitic infection, either an IFN-γ dominant type 1 or an IL-4 dominant type 2 immune response may effectively lead to resolution of infection. Future recombinant or nucleic acid vaccines will likely include genes for desirable cytokines in addition to protective antigens. Currently, vaccine development is in progress for *S. mansoni* in which novel delivery systems for IL-12 are being utilized to redirect the harmful type 2 immune response which causes severe immunopathology towards a host protective type 1 immune response (T. Wynn, personal communication). Targeting immunoregulatory molecules for vaccine development also could lead to the development of vaccine candidates for human

hookworm infection, in which the Th2 response is host-protective. However, understanding of the role of cell surface and secreted molecules required for the development of the protective Th2 response during hookworm infection remains rudimentary and further studies are required for vaccine development. In addition, the same effector T cells involved in host protection to hookworms also mediate immediate hypersensitivity and thus, elucidation of the molecules regulating the Th2 response to *H. polygyrus* may also provide the basis for the development of immunotherapies for allergy.

The type 2 immune response is characterized by rapid increases in T cell IL-4 production, intestinal mastocytosis, blood eosinophilia, serum IgE and IgG1 levels and GC formation. Initially our studies showed that T cell activation is blocked following the administration of the fusion protein, CTLA-4Ig. Using antibody intervention and gene knockout experiments, this dissertation extends these initial findings by characterizing individual functional roles for B7-1 and B7-2 on APCs, and CD28 and CTLA-4 on Th cells. Our findings in the *H. polygyrus* system include: 1) B7-1 and B7-2 are sufficient to initiate the type 2 immune response; 2) B7-2 is required to sustain Th2 cytokine production and elevations in serum IgE levels; 3) CD28 is required for T cell activation leading to Th2 cytokine production and effector function in wild-type mice; 4) CTLA-4 is not essential for initial T cell activation, but is required for T cell effector function; and 5) B7 ligand interactions are not necessary for memory T cell induction.

The implications for understanding allergy with a helminth model may have practical human benefits. Manipulating B7 ligand interactions may be an important target for developing therapies to promote the Th2 immune response to helminths and to block

the Th2 response during allergy. Recent clinical trials have suggested the efficacy of using CTLA-4Ig to treat psoriasis in the absence of generalized immunosuppression (Lebwohl et al., 1997). Our studies further examine the individual roles of B7-1/B7-2 and CD28/CTLA-4 during an immune response. Although considerably more research is required, they provide a framework for the development of future immunotherapies targeting specific members of this costimulation molecule pathway.

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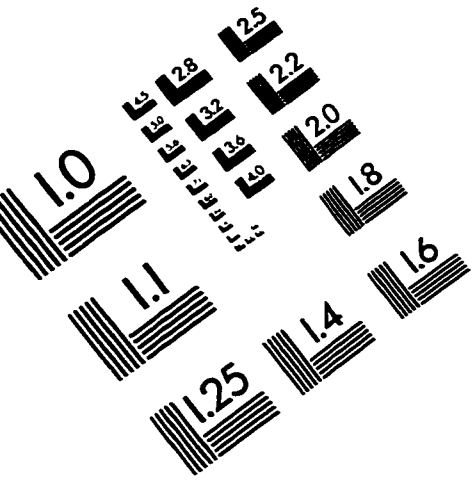
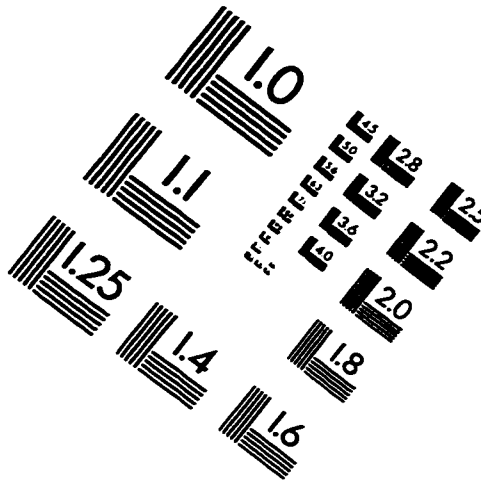
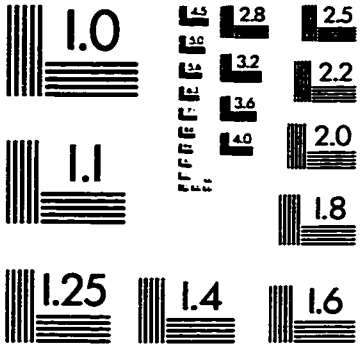
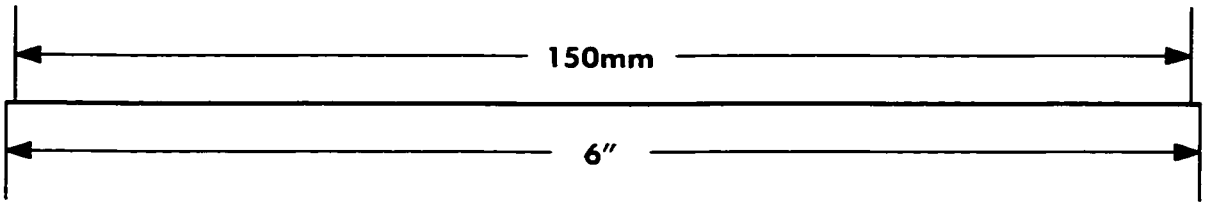
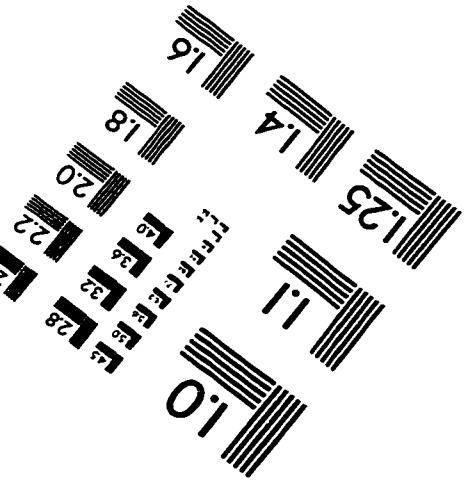
IMAGE EVALUATION TEST TARGET (QA-3)

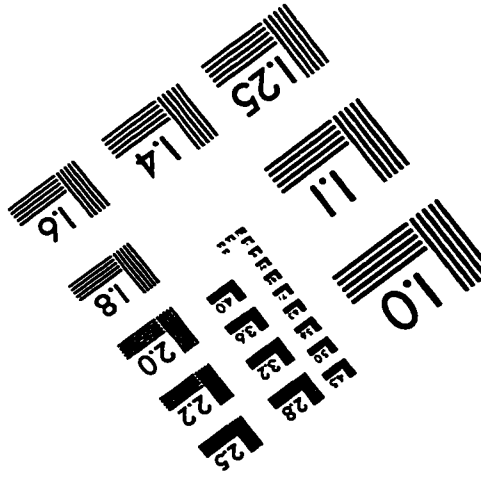












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